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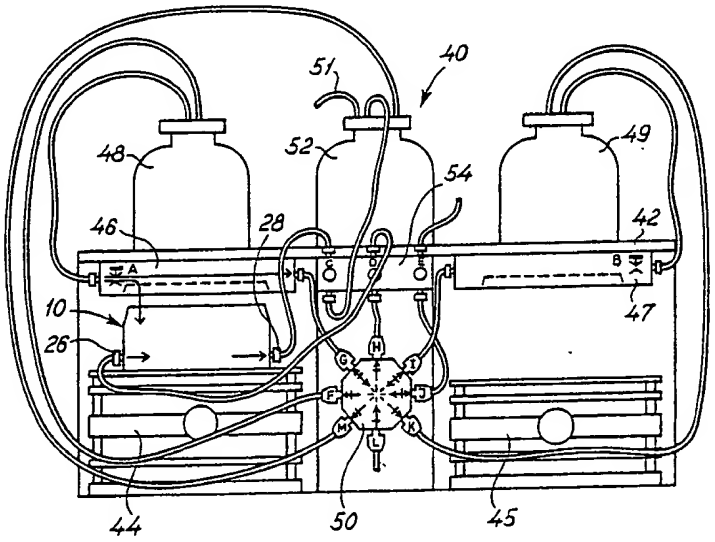
(54) Title: AN APPARATUS AND A METHOD FOR THE SYNTHESIS OF PEPTIDES

(57) Abstract

An apparatus for use in chemical synthesis, especially peptide synthesis, comprises a synthesis chamber unit having a multiplicity of synthesis chambers, each of which has a liquid inlet and a liquid outlet, means for introducing liquid into the individual synthesis chambers and means for simultaneous removal of liquid via the liquid outlets of the synthesis chambers by regulation of the fluid pressure difference between the liquid inlets and the liquid outlets. A method for peptide synthesis using such an apparatus comprises the provision in each of the synthesis chambers of a solid-phase support material having a first, at least N-protected amino acid coupled thereto, after which a liquid deprotection reagent is introduced into the synthesis chambers. Following deprotection, the deprotection reagent is removed, after which a second, at least N-protected amino acid is introduced into each synthesis chamber in order to couple the first and second amino acids. Third, fourth, etc. amino acids may be coupled analogously. Complete removal of the deprotection reagent from the synthesis chambers and the support material can be ensured by incorporating a stable, intensely coloured dye, e.g., azorubin, in the deprotection reagent. The apparatus and the method make possible the parallel synthesis of a large number of peptides, e.g. peptides having overlapping amino acid sequences and constituting part of a longer peptide chain. The apparatus can be adapted to manual, semi-automatic or fully automatic performance of the various steps in the synthesis procedure.



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AN APPARATUS AND A METHOD FOR THE SYNTHESIS OF PEPTIDES

FIELD OF THE INVENTION

The present invention concerns an apparatus and a method for parallel solid-phase synthesis of a multitude of peptides on a preparative (i.e. milligram or larger) scale, and it is particularly well-suited for the synthesis of a large number of peptides with overlapping amino acid sequences, each of which constitutes part of a longer peptide or protein chain, e.g., of an antigen from a pathogenic microorganism or a virus. Suitable embodiments of an apparatus according to the invention may be embodiments intended for manual, semi-automatic or fully automatic performance of the various steps in the synthesis procedure.

BACKGROUND AND DETAILED DESCRIPTION OF THE INVENTION

In the medical and biological sciences it is often of great importance to be able to carry out screening of substances which are known or presumed to be involved in a biochemical reaction. Such a reaction will typically be a reaction involving proteins and their interaction with other cellular components such as cell membranes, hormones or antigens, and in such cases the investigation will require a detailed knowledge of the molecular properties of the fragments, i.e., peptides, from which the proteins are built up; alternatively, it may, for example, be desirable to characterize the receptor peptide/ligand interaction by varying the composition of the ligand. It is thus of interest to be able to prepare a large number (e.g. 50 or more) analogous peptides or to be able to examine systematically the properties of various fragments of the amino acid sequence of a protein by the synthesis of a large number of peptides with overlapping amino acid sequences. The latter method will be applicable, for example, for the preparation of a monoclonal antibody repertoire of known specificity, or for the development of artificial vaccines free of side effects.

Current developments with regard to the synthesis of peptides or proteins are mainly directed towards further development and modifi-

cation of the strategy developed by R. B. Merrifield for solid-phase synthesis, solid-phase synthesis generally b ing far better than synthesis in homogeneous solution with respect to product yield and the amount of work necessary, and for automating the process of synthesis.

The methodology originally developed by Merrifield [see, e.g., *J. Am. Chem. Soc. 85*, 2149 (1963)] employs a functionalized cross-linked styrene/divinylbenzene copolymer as the solid phase support. This copolymer is normally provided in the form of spheres or particles, often with a predominant particle size of 20-80 μm. The functionalization originally preferred by Merrifield was functionalization of the aromatic rings of the copolymer with chloromethyl groups, but a number of other functionalities, including aminomethyl, α-aminobenzyl and α-amino-4-methylbenzyl, have subsequently been employed. Regardless of its nature, the purpose of the functionality is to provide an anchoring bond between the support and the C-terminal of the first amino acid which it is desired to couple to the support. More recent refinements of the Merrifield methodology include the further incorporation of a bifunctional "linker" group (also called a "spacer" or "handle" group) between a functionality (e.g. one of the above-mentioned functionalities) on the polystyrene chain of the support and the C-terminal of the first amino acid to be coupled, the reactivity of the linker being tailored, *inter alia*, to fulfil certain requirements with regard to the coupling of the first amino acid to the support and/or with regard to the ease with which the completed synthesized peptide chain is cleaved from the support.

The following abbreviations are used in the present specification and claims:

DMF:	*N*,*N*-dimethylformamide;
DMAP:	4-(*N*,*N*-dimethylamino)pyridine;
Dhbt:	3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl;
Dhbt-OH:	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine;
tBu:	*tert*-butyl;
Fmoc:	9-fluorenylmethyloxycarbonyl;
PTFE:	polytetrafluoroethylene;

Pfp: pentafluorophenyl;

TFA: trifluoroacetic acid;

HPLC: high performance liquid chromatography

The abbreviations used for the amino acids are those recommended by the "IUPAC-IUB Commission of Biochemical Nomenclature" [*J. Biol. Chem.*, *247*, 977-983 (1972)].

Owing to significant developments in molecular biology from the beginning of the 1970's, a need for a re-evaluation of solid-phase peptide synthesis methods arose, and it became evident that the reaction methodology developed by Merrifield was not necessarily optimal. These considerations led to the development of a new variant of a method for solid-phase peptide synthesis.

This new variant employs a polar polydimethylacrylamide support incorporating acryloylsarcosine methyl ester groups as functionalities to which suitable "linker" groups can be coupled. This polymer is permeated freely and solvated by a large number of solvents, including water, but especially by dipolar aprotic solvents of the dimethylformamide type. Furthermore, dimethylformamide is generally well-suited as a solvent for protected peptides, and is a preferred solvent for many peptide bond formation reactions.

An important aspect of this "polyamide" method for peptide synthesis is that the conventional acid-labile *t*-butoxycarbonyl protecting groups are replaced with other, base-labile *N*-protecting groups such as Fmoc, which is cleaved off in a few seconds upon treatment with secondary amines such as piperidine. At the same time the benzyl-based side-chain protecting groups employed in the traditional Merrifield method are replaced with, e.g., *t*-butyl-based side-chain protecting groups. A combination of protecting groups of the latter types makes solid-phase synthesis possible under considerably milder conditions than those employed in the Merrifield strategy, thereby broadening the range of applicability of the solid-phase method considerably.

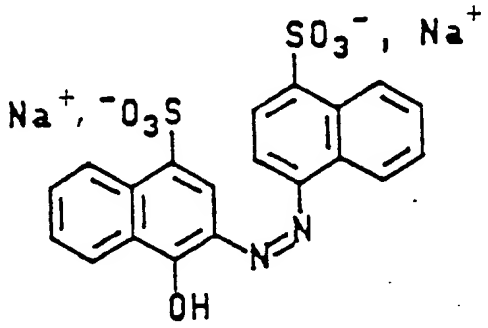
A general description of this "polyamide" strategy is given by Sheppard [*Chem. Brit.* 402 (1983)].

Useful designs f solid-phase peptide synthesis methods which employ this "polyamide" support are normally those in which the growing peptide chain is attached to the support during the synthesis by means of an acid- or base-cleavable "linker", e.g., 4-hydromethylphenoxyacetic acid, 3-methoxy-4-hydroxymethylphenoxyacetic acid (both of which are acid-cleavable) or 4-hydroxymethylbenzoic acid (which is base-cleavable). Thus, the methyl ester functions of the support are first reacted with ethylenediamine. The free amino groups in the ethylenediamine units are then acylated with the "linker", after which the first amino acid is coupled to the hydroxymethyl group of the "linker" in the form of an Fmoc-protected amino acid anhydride; the coupling process is catalyzed by DMAP. Alternatively, the first amino acid can be added to the support, after packing the latter in suitable columns, wells or other reaction containers, by the reaction of the Fmoc-protected amino acid Pfp ester catalysed by DMAP.

After establishing the support with the coupled, first, at least *N*-protected amino acid, the synthesis cycle continues with cleavage of the Fmoc group from the coupled, first amino acid followed by coupling of the next Fmoc-protected amino acid with its carboxyl group suitably activated, e.g. by introducing the Fmoc-protected amino acid in the form of a symmetrical anhydride or an activated ester.

A preferred embodiment of a method and an apparatus for solid-phase peptide synthesis according to the invention employs the above-mentioned "polyamide" solid-phase strategy. The above-mentioned Fmoc-protected amino acid anhydride/DMAP strategy is employed for coupling of the first amino acid, and piperidine dissolved in DMF is used as base for cleavage of the Fmoc group (i.e. deprotection) from this and from subsequent amino acids. Second, third, etc., amino acids to be coupled are introduced in the form of Fmoc-protected amino acid O-Dhbt esters dissolved in DMF. The latter Dhbt ester derivativ s have the great advantage that the liberated Dhbt-OH reacts with remaining amino groups present with the formation of the strongly

yellow-coloured anion Dhbt-O$^-$, which disappears gradually as the amino groups become acylated. The progress of the coupling process can thus easily be monitored. A detailed description of the chemistry involved in this "Dhbt indicator" reaction is given by Atherton et al. [*J. Chem. Soc. Perkin Trans. 1* (1988) 2887]. After cleavage of the Fmoc group from the last-coupled amino acid by treatment with base, e.g. piperidine, it is important that any remaining base is removed before introduction of the next amino acid to be coupled. For this purpose, in a further aspect of the method according to the invention a "deprotection reagent" incorporating a dye is used, washing of the base-treated support with its coupled amino acids being continued until all traces of the dye have disappeared from the reaction medium. In a preferred aspect of the method according to the invention the intensely red-coloured dye azorubin is preferred,



azorubin

this compound being stable towards the reagents used during the building up of the peptide chain, for example the solvent (DMF), which is also used as washing agent, and the mixture of DMF and piperidine which serves to deprotect the last-coupled *N*-protected amino acids. Other dyes will, however, be applicable. Such dyes include, e.g., azo compounds which are not base-sensitive, acylating or alkylating, although any dye with a suitable intense colour and corresponding properties will, in principle, be applicable.

There are presently three known methods for the solid-phase preparation of peptides by multi-synthesis. Geysen et al. [*Proc. Natl. Acad. Sci. USA, 81*, 3998-4002 (1985)] employ acrylic acid grafted poly-

ethylene sticks as support, and 96 microtiter wells with solutions of Fmoc-pr tected amino acid pentafluorophenyl esters for building up the peptide chains. The method is fast since the peptides are built up substantially simultaneously, but it lacks flexibility and has the limitation that only analytical amounts (micrograms) of peptide can be prepared. Houghten [*Proc. Natl. Acad. Sci. USA, 82*, 5131-5135 (1985)] has developed a method in which the support is a support of the traditional "Merrifield" type, i.e., a functionalized cross-linked styrene/divinylbenzene copolymer, which is divided into portions in sealed bags of polypropylene net, the reactions taking place in containers with the respective anhydrides of Boc-protected amino acids. The method suffers from lack of organization of the synthesis protocol, leading to a high probability of mistakes being made. Furthermore, *in situ* activation to unstable anhydrides is unpractical and the method is uneconomical. The third method has been developed by Dupont [Dupont: RaMPS™ Multiple Peptide Synthesis System] and is based on a parallel arrangement of 25 electrically driven shakers. The chemistry involved is the use of Fmoc-protected amino acid pentafluorophenyl esters on a traditional polystyrene support, and all 25 reaction chambers are treated individually. The method is uneconomical in use, and unnecessarily large amounts of peptides are prepared at one time. The small number and the large amount render the method unsuitable for screening on a large scale.

The present invention provides an apparatus for the synthesis of peptides, the apparatus being characterized by having a synthesis chamber unit in which there are one or more synthesis chambers, at the top of each of which there is a liquid inlet and at the bottom of each of which there is a liquid outlet, means for introducing liquid into the individual synthesis chambers via the liquid inlets, and means for simultaneous removal of liquid via the liquid outlets of the synthesis chambers by regulation of the fluid pressure difference between the liquid inlets and the liquid outlets. Using this apparatus, reactions can take place in all the synthesis chambers, these reactions being identical or different in the various chambers, and liquid can be removed from all the synthesis chambers simultaneously after the reactions are complete. Since the removal of liquid takes place by regulation of the fluid pressure difference between the

liquid inlets and the liquid outlets, the removal can take place without the use of valves placed in close proximity to the synthesis chambers and which could lead to contamination of the liquid reactants in the synthesis chambers with liquid from previous reactions.

The cross-sectional area of each of the liquid outlets is preferably considerably smaller than the cross-sectional area of the associated synthesis chamber, since liquid can then be prevented from running out through the liquid outlets by the establishment of a slight excess pressure in the liquid outlets. The liquid outlets may optionally have such small cross-sectional dimensions that the liquid cannot run out through the liquid outlets under the influence of gravity and the liquid can then be forced out through the liquid outlets by establishing an excess pressure above the liquid surface in the synthesis chambers. However, in preferred embodiments, the means for removal of liquid are adapted to alternately introduce gas under pressure to the liquid outlets in order to prevent the liquid from running out of the synthesis chambers, and to produce a vacuum in the liquid outlets, when the liquid is to be removed from the synthesis chambers. This alternation between introduction of gas under pressure and establishment of a vacuum can be achieved by connecting the liquid outlets of the synthesis chambers to a common outlet conduit which in turn is connected to a switching valve adapted to alternately connect the outlet conduit to a source of gas under pressure and a vacuum source, respectively.

Liquid chemical substances, reagents, washing liquids, etc., can be introduced into the synthesis chambers in any suitable manner. However, in a preferred embodiment the means for introducing liquid comprise a multipipette unit having a multiplicity of essentially parallel pipettes which are spaced apart from each other in a pattern corresponding to the pattern formed by the liquid inlets of the synthesis chambers, such that each pipette of the multipipette unit can be positioned opposite a liquid inlet of the synthesis chambers. A desired liquid can thus simultaneously be introduced into all the synthesis chambers or only a group of them by means of the multipipette unit, each pipette being positioned opposite a liquid inlet of the synthesis chambers in the group in question.

The means for introducing liquid can furthermore comprise a liquid-introduction unit having liquid-introduction ducts on its lower surface, the number of which liquid-introduction ducts corresponds to the number of synthesis chambers, the liquid-introduction ducts being arranged in a pattern corresponding to the pattern formed by the liquid inlets of the synthesis chambers and being adapted to be positioned opposite the liquid inlets of the synthesis chambers by relative movement of the synthesis chamber unit and the liquid-introduction unit, and the liquid-introduction ducts can be connected to a common liquid reservoir fashioned in the liquid-introduction unit. This liquid-introduction unit can advantageously be used to introduce one and the same liquid into all the synthesis chambers from the common liquid reservoir. Such a liquid-introduction unit can, by way of example, advantageously be employed for the simultaneous introduction of washing liquid into the synthesis chambers. The liquid-introduction unit and the synthesis chamber unit are suitably adapted to be moved away from each other when the liquid-introduction unit is not in use, so that the liquid-introduction unit does not obstruct other forms of liquid-introduction means, e.g. the previously mentioned multipipette unit.

The liquid reservoir can comprise a liquid cavity with a bottom surface and a downward-facing horizontal abutment surface. The liquid-introduction ducts can be in the form of tubes with essentially identically pointed upper ends and with identically inclined upper end surfaces, and the pointed upper ends of the tubes can be in contact with the horizontal abutment surface while the lower part of all the inclined end surfaces can be at a distance from and above the highest part of the bottom surface of the liquid cavity. This construction ensures that the tube openings delimited by the inclined upper end surfaces are all at exactly the same level and slightly above the bottom surface of the liquid cavity.

In the case of a variety of combinations of structural materials and solvents, the latter construction will ensure that liquid running into the liquid cavity will run downwards through all the above-mentioned tubes simultaneously. However, in the case of certain

combinations of structural materials and solvents, surface and/or other phenomena can lead to unsatisfactory results when employing the latter constructional principles for the liquid reservoir and liquid-introduction ducts. The preferred basic structural material for preferred embodiments (*vide infra*) of a synthesis chamber unit and a liquid-introduction unit according to the invention is PTFE, and the preferred reaction solvent and washing liquid (*vide infra*) is DMF; this combination of materials has been found to give rise to surface phenomena which under certain circumstances can lead to "creeping" of pure DMF, or solutions of other reagents in DMF, along PTFE surfaces in such a manner that satisfactory simultaneity of introduction of liquid into the various chambers of the synthesis chamber unit from the liquid-introduction unit is not achieved. For this reason, in an alternative preferred aspect of the invention the above-described combination of a downward-facing horizontal abutment surface of the liquid reservoir, and the tubes with essentially identically pointed upper ends and identically inclined upper end surfaces, the pointed upper ends of the tubes being in contact with the horizontal abutment surface, are replaced by a combination of (i) a downward-facing horizontal surface having uniform, substantially hemispherical depressions and (ii) uniformly dimensioned vertical tubes with upper end surfaces at a right angle to the tube axis, the upper ends of the tubes projecting uniformly up into the centres of the depressions, but not making contact with the inner surface thereof, and being at a distance from and above the highest part of the bottom surface of the liquid cavity.

Similarly, while there is no possibility of transfer of liquid from one reaction well of a synthesis chamber unit to another by capillary action in preferred embodiments of a synthesis chamber unit [owing to the size of the outlet conduit (*vide supra*)], in the case, e.g., of the combination PTFE/DMF there exists the possibility, particularly when adjusting the pressure conditions applying in the liquid outlets of the synthesis chambers, of transfer of liquid from one reaction well to another by means of the above-mentioned "creeping" of DMF along the lower PTFE surface of a synthesis chamber unit. For this reason, in alternative preferred embodiments of a synthesis chamber unit according to the invention the liquid outlets of the synthesis

chambers comprise tubes, preferably stainless steel tubes (*vide infra*), which are of small cross-sectional dimensions and whose lower openings (furthest from the synthesis chambers) project freely down into the common outlet conduit.

The invention also relates to a method for multiple peptide synthesis using the above-described apparatus, whereby there is provided in each of the synthesis chambers a solid-phase support material having a first, at least *N*-protected amino acid coupled thereto, and whereby a liquid deprotection reagent is introduced into the synthesis chambers by means of the liquid-introduction means, the reagent being removed from the synthesis chambers after completion of the reaction by means of the liquid-removal means, after which a second, at least N-protected amino acid is introduced into each of the synthesis chambers by means of the liquid-introduction means. Using the apparatus, different, pure peptides can thus be synthesized simultaneously, flexibly and economically in preparative quantities, since further at least *N*-protected amino acids can be introduced into the synthesis chambers, the introduction being preceded by introduction of deprotection reagent.

The apparatus and the method meet the growing demand for accelerated methods for the production of a large number of peptides in sufficient amounts and with a reasonable chain length. The amount of peptide (often approximately 1-50 mg of each peptide) prepared can be decided arbitrarily and will often be a compromise between - on the one hand - the desired amounts of peptides (e.g. for immunization experiments or affinity purification of receptors) and - on the other hand - economy and flexibility.

A dye can advantageously be added to the deprotection reagent. After removal of the deprotection reagent, and prior to the subsequent introduction of an amino acid in the synthesis chambers, the synthesis chambers are preferably washed through, a liquid washing agent being introduced by means of the liquid-introduction means and removed by means of the liquid-removal means. Owing to the presence of dye added to the deprotection reagent, effective washing with the washing agent can be ensured.

It will be apparent to a person skilled in the art that at least some of the principles underlying an apparatus and a method according to the present invention will be applicable in connection with the synthesis of other types of chemical compounds whose synthesis involves the repetition of certain reaction steps.

Similarly, the use according to the invention of a dye to indicate that adequate washing has taken place is not limited solely to peptide synthesis.

BRIEF DESCRIPTION OF THE DRAWING

The invention will be further explained in the following with reference to the drawing, in which

fig. 1 shows schematically a laboratory version of an apparatus according to the invention with a reaction block also in accordance with the invention,
fig. 2 shows schematically and partly in perspective a partially automated apparatus according to the invention,
fig. 3 shows in perspective and in cross-section the reaction block shown in fig. 1 positioned under an associated liquid-distribution manifold in the apparatus shown in fig. 1,
fig. 4 shows in perspective the reaction block shown in fig. 3,
fig. 5 shows a cross-section through a commercially available multipipette for use in connection with the reaction block according to the invention shown in fig. 4,
fig. 6 shows in perspective and partially in cross-section the reaction block shown in fig. 1, 3 and 4 and the associated liquid-distribution manifold shown in fig. 1,
fig. 7 and 8 show mutually perpendicular vertical cross-sections through the reaction block shown in fig. 4 and 6,
fig. 9 shows the lower part of the reaction block shown in fig. 6 as seen from above,
fig. 10 shows a tube of the liquid-distribution manifold according to the invention shown in fig. 11-13,

fig. 11 shows a vertical cross-section through a liquid-distribution manifold of the type shown in fig. 1, 3 and 6,

fig. 12 shows the liquid-distribution manifold shown in fig. 11 as seen from below,

fig. 13 shows a vertical cross-section at a right angle to the cross-section through the liquid-distribution manifold shown in fig. 11, showing the positioning of tubes as shown in fig. 10,

fig. 14 shows a vertical cross-section through a reaction vessel to which the support material on which the peptides are synthesized in the reaction block according to the invention is transferred prior to a last step in the synthesis procedure,

fig. 15 shows a cross-section at a right angle to the cross-section through the reaction vessel shown in fig. 14,

fig. 16 shows the reaction vessel shown in cross-section in fig. 14 and 15 as seen from above,

fig. 17 shows a reaction tray according to the invention for reagents which are transferred to the reaction block according to the invention shown in fig. 1, 3, 4, 6, 7, 8 and 9 by means of the multipipette shown in fig. 5,

fig. 18 shows a vertical cross-section through the reaction tray shown in fig. 17,

fig. 19 and 20 show mutually perpendicular vertical cross-sections through a further embodiment of a reaction block according to the invention,

fig. 21 shows a further embodiment of a liquid-distribution manifold according to the invention as seen from below, and

fig. 22 shows a vertical cross-section through the liquid-distribution manifold shown in fig. 21.

DETAILED DESCRIPTION OF THE DRAWING

Fig. 4 shows schematically and in perspective a reaction block, indicated by the reference numeral 10, for a peptide synthesis apparatus according to the invention, the reaction block preferably being made of PTFE and having 8 x 12 wells, of which a single well-opening on an upper rectangular surface of the reaction block is indicated by the reference numeral 12. The reaction block 10 is a box-shaped

rectangular block, the above-mentioned upper rectangular surfac of which has chamfered edges, one of which is indicated by the ref rence numeral 14. The chamfered edges serve to guide the reaction block relative to associated compon nts, e.g., during introduction of the reaction block in the apparatus shown in fig. 1 relative to a liquid-distribution manifold fixed in position in the apparatus and similarly relative to a reaction vessel shown in fig. 14-16, to which peptides synthesized in the total of 96 wells in the reaction block are transferred to individual chambers before a last step of a peptide synthesis process. The total of 96 wells of the reaction block 10 are spaced apart at a distance of 9 mm, which is standard for microtiter equipment (the distance between two neighbouring wells in a row, each containing 8 wells, being 9 mm, and the distance between two neighbouring wells in two different rows, of which there are 12 in all, likewise being 9 mm).

In fig. 6-9, the reaction block shown in fig. 4 is shown in greater detail. As seen from the lower part of fig. 6, the reaction block 10 consists essentially of two parts, an upper part indicated by the reference numeral 16 and a lower part indicated by the reference numeral 18, held together by screws. There are milled grooves or channels in the lower part 18, as seen from fig. 6 and fig. 9 which show the lower part 18 of the reaction block 10 seen from above. These milled grooves in the lower part 18 of the reaction block 10, one of which is indicated by the reference numeral 20, are in connection with two threaded holes indicated by the reference numerals 22 and 24, respectively, in which are fixed fittings indicated by the reference numerals 26 and 28, respectively, as seen from fig. 6, for establishing connection between the grooves or channels 20 and external tubing, as will be seen from the subsequent detailed description with reference to fig. 1. The milled grooves or channels in the lower part 18 of the reaction block 10 can either be put under vacuum or under pressure, as also explained below. The two threaded holes 22 and 24 are, as seen from fig. 8, connected to ducts indicated by the reference numerals 23 and 25, respectively, which connect the threaded holes 22 and 24 with the milled grooves or channels in the lower part 18 of the reaction block 10. The previously mentioned wells in

the reaction block 10 are fashioned in the upper part of the reaction block 10.

The individual wells consist, as seen from fig. 7 and 8, of tw bores, an upper bore indicated by the reference numeral 30 of relatively large diameter and a lower bore indicated by the reference numeral 32 of relatively small diameter, the bores being formed coaxially in prolongation of each other. In the bottom of the upper bore of relatively large diameter is placed a PTFE filter indicated by the reference numeral 34. The lower bore 32 forms a narrow outlet for the for upper bore of relatively large diameter of the above-mentioned well. The diameter of the upper bore 30 in the embodiment shown in fig. 7 and 8 is 6 mm, whilst the depth of the upper bore 30 is 25 mm. The diameter of the outlet-forming lower bore 32 is 1 mm, and the depth of the bore 32 is 5 mm.

Fig. 19 and 20 show cross-sections through an alternative preferred embodiment of a reaction block 10 for a peptide synthesis apparatus according to the invention, this alternative reaction block being made of PTFE and being particularly preferred for use in conjunction with DMF (or other suitable solvents which tend to "creep" on PTFE surfaces) as reaction solvent and washing liquid, and consisting, in the same manner as the reaction block shown in fig. 7 and 8, of an upper part 16 and a lower part 18, held together by screws. The lower part 18 in fig. 19 and 20 is essentially identical with that shown in fig. 7, 8 and 9, and the upper part 16 in fig. 19 and 20 differs from that shown in fig. 7 and 8 only with respect to the constructional details of the liquid outlets: as in fig. 7 and 8, each well has an upper bore 30 of relatively large diameter and a lower bore of relatively small diameter, the bores being formed coaxially in prolongation of each other; however, in the upper part 16 shown in fig. 19 and 20 there is inserted in the lower bore a tightly-fitting tube indicated by the reference numeral 201, preferably made of stainless steel, both open ends of which are cut at a right angle to the tube axis. The tube 201 in the lower bore forms a narrow outlet for the upper bore 30. The dimensions of the upper bore are as given for the embodiment shown in fig. 7 and 8. The diameter of the lower bore in which the tube 201, made of stainless steel, is inserted is ca.

1.5 mm and the depth is 5 mm. The outside diameter of the tube 201 is then also ca. 1.5 mm, and the internal diameter is ca. 1 mm. The total length of the tube 201 is 10 mm, of which 5 mm protrude below the plane of the underside of the upper part 16 of th reaction block 10.

The tube 201 may alternatively be made of PTFE, in which case the lower bore for the tube will necessarily be of somewhat larger diameter than mentioned above, in order to take account of the greater wall thickness required for a PTFE tube of adequate rigidity and with an internal diameter substantially equal to that of the above-described stainless steel tube. The use of PTFE as a material for the tubes 201 has been found not to give rise to any problems associated with "creeping" of DMF.

For the synthesis of peptides using the reaction block 10, the support, Macrosorb-SPR (polydimethylacrylamide, polymerized in kieselguhr which functions as a hard matrix; the polymer contains acryloylsarcosine methyl ester as the functional group), which is well-suited for column- or well-packing as in the apparatus according to the invention, is placed in the individual wells of the reaction block 10.

During synthesis the peptides will be attached to the support by means of an acid- or base-cleavable "linker" (e.g. 4-hydroxymethylphenoxyacetic acid). Thus, the methyl esters of the support are first reacted with ethylenediamine. The free amino groups are then acylated with the linker, the first amino acid then being introduced on the hydroxymethyl group of the linker using Fmoc-protected amino acid anhydride and DMAP catalysis. Alternatively, the first amino acid can be introduced after well-packing by reaction with Fmoc-protected amino acid Pfp esters catalyzed by DMAP.

The peptide synthesis apparatus according to the invention, or rather a laboratory embodiment thereof, will be described in more detail in the following with reference to fig. 1. The apparatus as a whole is indicated by the reference numeral 40 and has a frame indicated by the reference numeral 42, which is preferably made of stainless steel

and which delimits a left-hand section, a middle section and a right-hand section. The construction of the left-hand section is such that it is a mirror image of the right-hand section, the left- and right-hand sections being designed for introduction of two different liquids into the individual wells in a reaction block mounted in the section in question. In fig. 1, the block 10 is thus shown placed into the left-hand section of the apparatus and mounted on a lifting table indicated by the reference numeral 44. In the right-hand section of the apparatus there is a lifting table indicated by the reference numeral 45 corresponding to the lifting table 44. By means of the lifting tables 44 and 45, a reaction block placed in the section in question can be lifted to an active position, in which the upper part of the reaction block has been guided by the previously described chamfered edges into a liquid-distribution manifold indicated by the reference numeral 46 in the left-hand section and indicated by the reference numeral 47 in the right-hand section of the apparatus. The liquid-distribution manifolds 46 and 47 shown in fig. 1 are also shown in fig. 6 and will be described in more detail with reference to fig. 10-13. On the upper part of the frame 42 in the left-hand section is placed a bottle-shaped container indicated by the reference numeral 48, and in the right-hand section of the apparatus is similarly placed a bottle-shaped container indicated by the reference numeral 49. The bottle-shaped containers 48 and 49 contain the above-mentioned two liquids. In the middle section of the apparatus there is a waste container indicated by the reference numeral 52, and a valve unit indicated by the reference numeral 50, which serves to establish connection between two or more inlets or outlets of the unit and to which inlets or outlets is attached tubing leading to the various components of the apparatus, including the bottles 48 and 49, the waste container 52 in the middle section of the apparatus, the liquid-distribution manifolds 46 and 47, the fittings 26 and 28 of the reaction block 10, and a reduction valve unit indicated by the reference numeral 54 with reduction valves C, D and E. The waste container 52 has tubing indicated by the reference numeral 51 connected to a vacuum source. Tubing leads from the reduction valve 54 E to the atmosphere, whilst the valve unit 50 has an inlet L connected to a pressure source for introducing nitrogen under pressure. In the liquid-distribution manifold 46 there is a shut-off

valve A, and the liquid-distribution manifold 47 also has a shut-off valve B. The bottle-shaped containers 48 and 49 are connect d by tubing to connections F and K, respectively, on the valve unit 50, whilst the ends of the liquid-distribution manifolds 46 and 47 opposite the above-mentioned shut-off valves A and B, respectively, are connected to connections G and I, respectively, on the valve unit 50. Connections H and J on the valve unit 50 are connected to the above-mentioned reduction valves D and E, respectively, on the reduction valve unit 54.

The above-mentioned liquid-distribution manifolds 46 and 47 and components of these are shown in detail in the upper part of fig. 6 and also in fig. 10-13. As seen from fig. 6, each liquid-distribution manifold comprises a central block indicated by the reference numeral 56, shown in three mutually perpendicular cross-sectional views in fig. 11, 12 and 13, as well as two end parts indicated by the reference numerals 58 and 60, respectively. The central block 56 has inner inclined surfaces corresponding to the chamfered edges of the reaction block 10, and the end parts 58 and 60 similarly have inner inclined surfaces corresponding to the chamfered edges of the reaction block 10. Each liquid-distribution manifold 46, 47 is held together by screws, as seen from fig. 6.

In each end part 58, 60 there is a fitting or a tubing connection indicated by the reference numerals 62 and 64, respectively. These fittings or tubing connections 62 and 64 are connected via respective bores in the end parts 58 and 60 to an elongated opening, of which fig. 6 only shows the elongated opening indicated by the reference numeral 66 of the end part 58. In the end part 60 there is a corresponding elongated opening. The opening 66 and the corresponding opening of the end part 60 are in direct connection with through-going bores indicated by the reference numeral 68 in the block 56 when the end parts 58 and 60 and the central block 56 are assembled. One of these through-going bores is indicated by the reference numeral 68 in fig. 6. The same through-going bore is shown in fig. 11-13. From the underside of block 56 a series of stainless steel tubes with chamfered or obliquely cut ends extend into the through-going bores 68, one such tube indicated by the reference numeral 70 being

shown in fig. 10. The positioning of the latter tubes in the through-going bores 68 in the bl ck 56 is seen from fig. 11 and 13. The tubes 70 thus serve to transport liquid introduced through one of the tubing connections 62 and 64 downwards through themselves and out through the opening in the tubes in question opposite the obliquely cut end. Owing to the very precise, identical cutting of all the tubes and the identical positioning of all the tubes in the through-going bore 68 in the block 56, simultaneous withdrawal of liquid from the block 56 takes place when the liquid level in the through-going bores reaches a height corresponding to that of the inclined openings in the tubes. As mentioned above, for numerous combinations of structural materials and solvents (e.g. aqueous solvents) this design of the liquid-distribution manifold with obliquely cut tubes will enable extremely convenient and suitable simultaneous washing of or addition of liquid to the 96 wells of the reaction block 10 controlled by means of only single shut-off valves A and B in the liquid-distribution manifolds 46 and 47, respectively. In these cases, the design of the liquid-distribution manifolds 46 and 47 ensures that addition of liquid to the liquid distribution manifold in question results in withdrawal of liquid through all the tubes, and thus transfer of liquid to all the wells in a reaction block mounted in the liquid-distribution manifold, upon introducing liquid into the liquid-distribution manifold in question.

Fig. 3 shows in perspective and in cross-section the reaction block 10 lowered slightly away from an associated liquid-distribution manifold 46 or 47 in the apparatus shown in fig. 1. The design of the through-going bores of the liquid-distribution manifold, the design of the associated tubes, as well as the design of the lower narrow openings of the individual columns or wells in connection with the machined or otherwise fashioned grooves or channels in the reaction block 10, are seen from fig. 3.

Fig. 21 and 22 show an alternative preferred embodiment of a liquid-distribution manifold 46, 47 for a peptide synthesis apparatus according to the invention, this alternative liquid-distribution manifold being particularly preferred for use in conjunction with DMF (or other suitable solvents which tend to "creep" on PTFE surfaces) as

reaction solvent and washing liquid. As seen from these figures, each liquid-distribution manifold comprises an upper part, indicated by the reference numeral 210, and a lower part, indicated by the reference numeral 212. As seen from fig. 21 and 22, the upper part 210 has threaded holes indicated by the reference numeral 211 for the attachment of the liquid distribution manifold to the frame of a peptide synthesis apparatus according to the invention, such as to the underside of the upper part of the frame 40 in the apparatus shown in fig. 1, by means of screws. The lower part 212 has inner inclined surfaces indicated by the reference numeral 214 corresponding to the chamfered edges of an associated reaction block 10. A sealing gasket indicated by the reference numeral 216, preferably made of hard silicone rubber or another suitable elastomer material, is sandwiched between the upper and lower parts. The length and breadth of the sealing gasket 216 match those of the liquid-distribution manifold, and a central rectangular opening in the gasket establishes a reservoir or cavity indicated by the reference numeral 218 for liquid when the upper and lower parts of the liquid-distribution manifold are assembled. The upper and lower parts of the liquid-distribution manifold 46, 47 are held together by screws indicated by the reference numeral 220 which pass through appropriate holes in the gasket 216.

At each end of the underside of the lower part 212 there is a threaded hole indicated by the reference numeral 222 for attaching a fitting or tubing connection. These threaded holes 222 are connected, via respective bores indicated by the reference numeral 224 and cut-out openings indicated by the reference numeral 225 (shown in fig. 21 by dashed lines) in the sealing gasket 216, to the above-mentioned liquid reservoir 218. From the underside of the lower part 212 of the liquid-distribution manifold 46, 47 a series of stainless steel tubes indicated by the reference numeral 70, both open ends of which are cut at a right angle to the tube axis, extend into the liquid reservoir or cavity 218. The 8 x 12 arrangement (standard for microtiter equipment) of the tubes 70 is shown schematically in fig. 22, the majority of the tubes having been omitted in this figure for simplicity.

The underside of the upper part 210 of the liquid-distribution manifold 46, 47 has a series of milled or machined, substantially hemispherical depressions indicated by the reference numeral 226 positioned in a pattern corresponding to that of the stainless steel tubes 70 in the lower part 212 of the liquid-distribution manifold. When the upper and lower parts of the liquid-distribution manifold and the associated sealing gasket 216 are assembled, the uppermost end of each stainless steel tube 70 thus projects up into the centre of a corresponding hemispherical depression 226, but does not make contact with the inner surface of the depression. The depth of the depression 226 is ca. 4 mm, the thickness of the sealing gasket is ca. 3 mm and the stainless steel tubes 70 are of a length such that 5 mm of each tube protrudes below the adjacent lower surface of the lower part 212 and the upper end of each tube extends into the associated depression 226 to a depth of ca. 2 mm. The internal diameter of the tubes 70 is ca. 0.5 mm, and the outside diameter is ca. 0.8 mm.

The tubes 70 thus serve to transport liquid introduced through a fitting or tubing connection in one of the threaded holes 222 downwards through themselves and out through the lower openings in the tubes. Owing to the identical dimensions and relative positioning of the depressions 226, and the identical dimensions and relative positioning of all the tubes 70, simultaneous withdrawal of liquid from the reservoir 218 takes place when the liquid level in the depressions 226 reaches a height corresponding to that of the upper openings in the tubes 70.

Four machined, identical, essentially semicircular depressions indicated by the reference numeral 228 in, and extending outwardly slightly beyond, the corners of the rectangle formed by the inner inclined surfaces 214 of the lower part 212 serve to permit free pressure equalization or air-venting upon the introduction of liquid from the liquid reservoir 218 via the tubes 70 into the wells of an associated reaction block 10 positioned in the active position with its chamfered edges in contact with the inner inclined surfaces 214 of the lower part 212 of the liquid-distribution manifold 46, 47.

It has been found that this embodiment of a liquid-distribution manifold enables convenient, simultaneous and highly uniform washing of the 96 wells of an associated reaction block 10 with DMF or addition of DMF-based liquid reagents to the wells, and this embodim nt is particularly well-suited for use in conjunction with the embodiment of a reaction block 10 which is shown in, and described with reference to, fig. 19 and 20.

The construction of this embodiment of a liquid-distribution manifold also substantially eliminates any possibility of air pockets being formed within the liquid reservoir 218, any formation of air pockets leading to a risk of non-uniformity of liquid delivery via the individual tubes 70. The construction has also been found to substantially eliminate any risk of certain individual tubes 70 delivering liquid at a significantly greater rate than others.

This preferred embodiment of a liquid-distribution manifold shown in fig. 21 and 22, and, optionally, the embodiment of a reaction block shown in fig. 19 and 20, may be employed in an apparatus for peptide synthesis according to the invention, such as the apparatus shown in fig. 1 or fig. 2, instead of those shown in, and described with reference to, fig. 3, 6 and 7-13 without any significant modifications of the remaining components of the apparatus.

As will be apparent from the above, it is important that especially the liquid-distribution manifolds in an apparatus according to the invention are substantially perfectly horizontal, in order to ensure correct and simultaneous introduction of liquid via the tubes 70 into the wells of a reaction block. For this purpose, the frame of an apparatus such as that shown in fig. 1 or 2 may suitably be equipped with, e.g., levelling screws, and the plate or other means to which the upper surfaces of the manifolds are attached may be equipped with a spirit level.

A synthesis cycle is then initiated by cleaving off the Fmoc group from the first-coupled Fmoc-protected amino acid (*vide supra*) by addition, from the container 49 via the right-hand liquid-distribution manifold 47, of a 20% solution of piperidine in DMF containing

0.01% of the dye azorubin. The dye indicates both that deprotection is taking place in all the wells and that the subsequent washing has been adequate. The washing takes place by the introduction of DMF from the left-hand container 48 via the left-hand liquid-distribution manifold 46. Preactivated Fmoc-protected amino acid O-Dhbt esters dissolved in DMF are used for coupling of amino acids to the growing peptide chain. The coupling reaction is monitored visually, since liberated Dhbt-OH reacts with remaining amino groups with the formation of the strongly yellow-coloured anion Dhbt-O^-, which disappears gradually as the amino groups become acylated.

If desired, the containers 48 and 49 in the laboratory version of an apparatus shown in fig. 1 for dispensing DMF and piperidine/DMF, respectively, to the respective liquid-distribution manifolds 46 and 47, and which are connected to a source of gas under pressure, may be replaced by suitable autoburettes or similar dispensing means which may be operated manually or by other means, and which can dispense the desired volumes of the liquids in question to the liquid-distribution manifolds.

After coupling of an amino acid, the support is washed with DMF, whereby all reagents are washed out. The amino-protecting group (the Fmoc group) is then cleaved off with 20% piperidine in DMF, and the synthesis cycle is complete.

All the wells are simultaneously washed with DMF or treated with piperidine. DMF or piperidine are added from above and are removed by suction from the bottom of the well. The individual solid, activated, protected amino acids are measured out with a metering spoon into a reagent tray, which will be described below with reference to fig. 17 and 18, and they are dissolved in DMF and added to the wells in dissolved form by pipetting with a multipipette, such as a multipipette indicated by the reference numeral 80 and shown in fig. 5, the latter being of the type Capp Multichannel Pipette manufactured by the firm Cappelen Laboratory Technics, Odense, Denmark. By establishing an excess pressure of nitrogen in the channels under the wells in the reaction block, the solutions in the individual wells do not run out of the wells, and they only run out when suction

is applied. The excess nitrogen pressure and suction are established via the valve unit 50 and the reduction valve unit 54. Dbht esters have been chosen for the coupling step, but the apparatus 40 can be used with other coupling methods without fundamental modifications. The Dbht method has the advantage that the reagent is self-indicating, since a yellow colour develops during the reaction and disappears when coupling is completed. Coupling times are typically about 2 hours. It is imperative to remove all piperidine before the next step in the synthesis, and the apparatus is thus constructed in such a manner that the piperidine treatment and the general washing take place in two separate steps. The half-life for the cleaving off of the Fmoc group is about 6 sec., but it is preferable to carry out a double piperidine treatment lasting a total of 10 min., which ensures complete reaction.

The last step in the peptide synthesis process is the cleaving off of the peptide from the support with 95% aqueous TFA. It is not practical to carry this out directly in the apparatus in which the synthesis takes place, since TFA is harmful to the steel parts of the construction. This operation is therefore performed in a separate reaction vessel indicated by the reference numeral 90, which is shown in fig. 14-16 and which is constructed in such a manner that all 96 support-bound peptides are transferred at the same time to a new set of 96 wells. The peptide is liberated in these wells by TFA treatment, and the peptides can be isolated, e.g. by simple pipetting with a multipipette, such as the multipipette 80 shown in fig. 5, to a further reaction vessel which is identical with the reaction vessel 90 and in which the TFA is forcibly evaporated simultaneously by 96 nitrogen streams.

As previously mentioned, the reaction vessel 90 has 96 wells positioned in correspondance to the wells of the reaction block 10 and is chamfered in such a manner that there remain 4 small edges which are complementary to the chamfered edges of the reaction block 10. Fig. 14 shows a vertical cross-section through the reaction vessel, a single well in the reaction vessel 90 being indicated by the reference numeral 92. Fig. 15 shows a vertical cross-section at a right angle to the cross-section in fig. 14, likewise showing the well 92,

whilst fig. 16 shows the reaction vessel 90 seen from above, the opening of the well 92 thus being visible.

Since more commonplace plastic materials cannot tolerate prolonged contact with DMF or piperidine, a number of components of the apparatus, including the tubing of the apparatus, the valve units 50 and 54, the block 10, the liquid-distribution manifolds 46 and 47 and the reaction vessel 90 are made of PTFE. The containers 48 and 49, as well as the waste bottle 52, are suitably made of glass or PTFE. The previously mentioned tubes or washing tubes 70 in the preferred embodiment are made of stainless steel.

The apparatus 40 is especially well-suited to the preparation of overlapping peptides from a long peptide or protein sequence, e.g. of an antigen from a pathogenic microorganism or a virus, or to the synthesis of analogous peptides. For the synthesis, the activated amino acids are placed in individual reagent chambers in the order in which they occur in the antigen.

Fig. 17 and 18 show a reagent tray indicated by the reference numeral 100 seen from above and in vertical cross-section, respectively, the reagent tray being especially well-suited for use in connection with the synthesis of peptides using the apparatus 40 according to the invention and further using a multipipette such as the multipipette 80 shown in fig. 5. The reagent tray contains a number of reagent chambers indicated by the reference numeral 102 in which the activated amino acids are present. The number of reagent chambers for a single reagent tray 100 can of course vary, but the individual reagent chambers 102 are preferably at a distance from a neighbouring chamber such that the individual pipette tips on the multipipette 80, shown in fig. 5, can be introduced into each reagent chamber 102 in the reagent tray 100, just as they can be introduced into the individual wells in the reaction block 10 for delivery of activated amino acids, previously sucked up from the respective reagent chambers 102 in the reagent tray 100, to the respective wells in the reaction block 10. The distance between the individual reagent chambers 102 in the reagent tray 100 is thus preferably equal to the distance between neighbouring wells in the reaction block 10. Fur-

thermore, the individual reagent chambers are preferably of a width such that all the pipette tips on the multipipette shown in fig. 5, which is used in connection with the r agent tray 100, can be introduced into a single reag nt chamber. The reagent tray 100 is preferably made of PTFE, like the previously described equipment, or of another material, such as stainless steel, which tolerates contact with the reactive reagents used in connection with peptide synthesis.

The reagent tray or container is, as will be understood, built up like a series of successive chambers, the distance between each chamber corresponding to the distance in the multichannel pipette 80. The individual amino acids are thus introduced eight at a time into individual wells, the dimensions of the reaction block likewise being adapted to the multichannel pipette. In each of the total of 96 wells there is thus introduced one (optionally different) amino acid. For the next addition the multichannel pipette is moved one chamber forward, whereby well no. 1 receives amino acid no. 2, well no. 2 receives amino acid no. 3, etc. With e.g. 6 coupling steps it can thus be seen that 96 different hexapeptides, e.g. overlapping sequences of part of an antigen, are built up. For coupling of one single amino acid, the reagent tray can thus have 96 chambers. For the building up of di-, tri-, tetra-, penta-, etc., peptides there must thus be 97, 98, 99, 100, etc. chambers. This can optionally be achieved with 4 x 26 chambers, placed one after another in a steel tray adapted thereto. 96 different nonapeptides can thus be built up, but this can of course be increased by the use of an extra reaction tray, etc. The method will likewise be very well-suited to the preparation of a large series of peptides in which, e.g., one or more amino acids are systematically replaced.

All 96 couplings are thus carried out at one time, although it will be the slowest reacting amino acid that determines the termination of a coupling cycle. The method will, however, be extremely fast, and in the space of a few days it will be possible to prepare the above-mentioned 96 hexapeptides in amounts of up to ca. 20 mg of each.

It will be possible to carry out the synthesis on the basis of simple instructions, no special expertise being required.

The use of the preferred embodiment of an apparatus as shown in fig. 1 and a method according to the invention for the synthesis of a peptide is further illustrated by the following example, in which the performance of the apparatus was examined by synthesizing one and the same pentapeptide in all 96 wells:

EXAMPLE 1

Synthesis of vitamin K dependent carboxylation substrate, H-Phe-Leu-Glu-Glu-Val-OH.

(a) Functionalization of the support

To Macrosorb-SPR (a copolymer of dimethylacrylamide and acryloylsarcosine methyl ester, polymerized in kieselguhr so as to provide a rigid matrix; 0.25 mmol methyl ester group/g) was added a large excess of ethylenediamine, after which the mixture was left for 48 hours. Excess ethylenediamine was removed by filtration and washing with DMF, until the washings gave a negative ninhydrin reaction, after which 2 equivalents of 4-hydroxymethylphenoxyacetic acid Dbht ester were added. After disappearance of the yellow colour of Dbht-O$^-$, the thus-functionalized support was filtered off, washed with DMF and with diethyl ether, and dried *in vacuo*.

(b) Building up of the pentapeptide

Fmoc-Val (10 equivalents) was initially coupled to the functionalized support (by conversion of Fmoc-Val to its symmetrical anhydride using diisopropylcarbodiimide in dichloromethane; after removal of the dichloromethane and redissolving in DMF the anhydride is reacted with the support in the presence of DMAP), after which the procedure was as follows:

25 mg of Fmoc-Val-support was measured out with a metering spoon and packed in each of the 96 wells, after which the support was washed, treated twice with an excess of a 20% (w/w) solution of piperidine in

DMF containing 0.01% (w/v) of azorubin (for a total of 10 min.) and washed with DMF until the red colour had disappeared.
Fmoc-Glu(tBu)-O-Dbht (3 equivalents) was added to all the wells from the reaction tray with a multipipett , th N_2 pressure in the underlying channels being adjusted as necessary. A total of 1 g of Fmoc-Glu(tBu)-O-Dhbt in 15.2 ml of DMF was used. Washing with DMF was carried out after disappearance of the yellow colour in all the wells.

The process was repeated analogously for the subsequent amino acids in the sequence (ca. 3 equivalents; a total of 1 g of Fmoc-protected amino acid O-Dbht ester in ca. 15 ml of DMF) and the synthesis was terminated by piperidine treatment, washing with DMF, washing with ether and drying. All 96 portions of the support were transferred at one time to the 96 wells in a reaction vessel and treated with 95% aqueous TFA (200 µl/well) for 2 hours. The solutions were pipetted into individual wells in a third tray, and each sample of support was washed with a further 100 µl of 95% aqueous TFA, which after removal by pipetting was combined with the first portion of TFA solution. The 96 individual solutions were evaporated almost to dryness using a multichannel stream of nitrogen. The peptides were washed individually with diethyl ether and analyzed by HPLC (RP C_{18}, 25 cm; solution A: 0.1% (v/v) aqueous trifluoroacetic acid; solution B: 10% (v/v) A in acetonitrile; gradient elution with from 20 to 60% (v/v) B in A over a period of 12 min.; the peptide has a retention time of 8.0 min.), and they gave 96 almost identical HPLC chromatograms. The sample of peptide from a single well was isolated quantitatively and subjected to gel filtration. The yield after gel filtration was 3.9 mg (98%). An amino acid analysis of this sample gave the following results (mol amino acid/mol Leu): Glu 1.98, Val 1.01, Leu 1.00, Phe 1.01.

Use of the apparatus 40 shown in fig. 1 using liquid-distribution manifolds as shown in fig. 21 and 22, and a reaction block as shown in fig. 19 and 20

1. The solvent (dimethylformamide, DMF) is forced into the left-hand liquid-distribution manifold 46 by means of a slight excess pressure. The DMF then runs through the 96 thin steel tubes 70 in the liquid-distribution manifold into the 96 wells of the reaction block 10. At this point the reaction block 10 is positioned in the active position under the liquid-distribution manifold 46 by means of the left-hand lifting table 44. The construction and positioning of the liquid-distribution manifold 46 results in simultaneous emptying of liquid through all 96 stainless steel tubes 70. The valve C is in the open position, thus allowing DMF to run out through a small outlet 201, in the form of a steel tube, in the bottom of each of the wells in the reaction block. Washing with DMF can be carried out to the required extent, and finally first valve A and then valve C are closed.

2. The lifting table 44 is lowered, and the reaction block 10 is placed on the other lifting table 45 and raised so as to position it in the active position under the right-hand liquid-distribution manifold 47. A 20% solution of piperidine in DMF is forced, via the valve B, by a slight excess pressure into the right-hand liquid-distribution manifold 47 and out through the 96 thin steel tubes 70. Excess piperidine solution is sucked out through the bottom of the wells. A new portion of piperidine solution is then added; after standing for 10 min., excess piperidine is sucked off, the lifting table 45 is lowered, and the reaction block 10 is again placed on the left-hand lifting table 44 and washed free of piperidine with pure DMF as described under 1.

In fig. 2 is shown a partially automated apparatus based on the ideas and principles underlying the invention. The whole apparatus shown in fig. 2 is indicated by the reference numeral 140 and includes the two-bottle shaped containers 48 and 49 and the waste container 52, just lik the apparatus 40 shown in fig. 1. In the apparatus 140 shown in fig. 2, the reaction block 10 is likewise positioned on a lifting table indicated by the reference numeral 144, the latter

being a motor-driven lifting table, the motor of which is indicated by the reference numeral 145. Instead of m ving the reaction block from one section of the apparatus to another section as described above, e.g. from the above-described left-hand section of the apparatus 40 shown in fig. 1 to the right-hand section shown in the same figure, the two liquid-distribution manifolds of the apparatus 140 shown in fig. 2 are mounted on a movable sledge arrangement in such a manner that the liquid-distribution manifolds 46 and 47 of the apparatus can be positioned in turn above the reaction block 10, which is then raised by means of the motor 145 of the lifting table 144. The liquid-distribution manifolds 46 and 47 are mounted on a common supporting plate indicated by the reference numeral 148 with which a driving wheel indicated by the reference numeral 149 of a motor indicated by the reference numeral 150 engages. Furthermore, the plate 148 moves between rollers, one of which is indicated by the reference numeral 151 in fig. 2. The position of the plate 148 is detected by means of either inductive or capacitive detectors or by an optical detector, indicated by the reference numeral 152 in fig. 2. The position of the reaction block 10 is similarly determined using position-detecting means, such as a light source indicated by the reference numeral 153 in conjunction with a light-detecting device indicated by the reference numeral 154. The three containers 48, 49 and 52 are connected to respective sections of a solenoid-controlled valve unit 50, which like the above-described motors 145 and 150, and the above-described position-detecting means 152, 153 and 154, are connected to a central controlling device in the apparatus 140. This central controlling device preferably consists of a CPU, which is contained in a section indicated by the reference numeral 160 of the apparatus and which operates in conjunction with a display device indicated by the reference numeral 162 with associated keyboard indicated by the reference numeral 164, as well as with an interface indicated by the reference numeral 166, which couples together the central controlling device 160 and means of the apparatus such as the above-described position-detecting means 152, 153, 154, and the motors 145 and 150.

The apparatus 140 shown in fig. 2 further incorporates an automated amino acid delivery device, which is placed in the left-hand side of

fig. 2. Another reaction block 10 is mounted on a support indicated by the reference numeral 170 in a car fully determined position. The reaction bl ck 10 is thus located in a recess in the upper surface of the support 170 or is held in a given, previ usly determined p sition relative to the support 170 by means of fixation means, which can, e.g., comprise pegs or projections protruding from the upper surface of the support 170 and which fit into corresponding holes or recesses in the bottom of the reaction block 10. The activated amino acids are stored in a unit indicated by the reference numeral 180 which consists of a series of reagent chambers from which an activated amino acid is transferred from the appropriate reagent chamber to a common conduit indicated by the reference numeral 182 via valves which can be operated by remote control. The unit 180 may, by way of example, be constructed in essentially the same manner as the reagent unit described in US Patent No. 4,746,490. From conduit 182 the activated amino acid in question is transferred to a guided delivery device indicated by the reference numeral 184 in an XY table, the guided delivery device having a three-way valve which can be operated by remote control. The delivery device 184 is mounted in a fixed position on an angular holder indicated by the reference numeral 186, which in turn is mounted in a fixed position on a fork-shaped support or carrier indicated by the reference numeral 188 protruding from the support 170. The delivery device 184 is moved relative to the support 170 by controlling and monitoring the position of the fork-shaped support 188 relative to the support 170 by means of drive means and position-determining means which are known *per se* and which are controlled by the central controlling device or CPU 160 of the apparatus. Any chosen activated amino acid contained in a reagent chamber in the reagent unit 180 can thus be transferred to a given well in the reaction block 10. The order in which the synthesis takes place is determined by the person operating the apparatus, who keys in the order of synthesis using the keyboard 164 of the apparatus. The functioning of the apparatus, including the order of synthesis, etc., can be monitored on the display device or display 162. The latter display 162 can inform the operator as to how far a reaction taking place in the right half of the apparatus 140 shown in fig. 2 has progressed.

The central controlling device or CPU 160 of the apparatus can be pre-programmed for automatic control of the washing or delivery operations and can be programmed with logical blocking to prevent incorrect operation of the apparatus by the operator.

CLAIMS

1. An apparatus for use in chemical synthesis, especially peptide synthesis, c mprising

a synthesis chamber unit having a multiplicity of synthesis chambers, at the top of each of which there is a liquid inlet and at the bottom of each of which there is a liquid outlet,

means for introducing liquid into the individual synthesis chambers via the liquid inlets, and

means for simultaneous removal of liquid via the liquid outlets of the synthesis chambers by regulation of the fluid pressure difference between the liquid inlets and the liquid outlets.

2. An apparatus according to claim 1, wherein the cross-sectional area of each of the liquid outlets is considerably smaller than the cross-sectional area of the associated synthesis chamber.

3. An apparatus according to claim 1 or 2, wherein the means for removing liquid are adapted to alternately introduce gas under pressure to the liquid outlets and to produce a vacuum in them.

4. An apparatus according to any of the claims 1 to 3, wherein the synthesis chamber unit is in the form of a block-shaped body and the synthesis chambers are in the form of upwardly open depressions or wells in the block-shaped body.

5. An apparatus according to any of the claims 1 to 4, wherein the liquid outlet in each of the synthesis chambers is optionally covered by a filter, preferably a plastic filter, placed in the bottom of the synthesis chamber.

6. An apparatus according to any of the claims 3 to 5, wherein the liquid outlets of the synthesis chambers are connected to a common outlet conduit which in turn is connected to a switching valve adapted to alternately connect the outlet conduit to a source of gas under pressure and a vacuum source, respectively.

7. An apparatus according to any of the claims 1 to 6, wherein the means for introducing liquid comprise a multipipette unit having a multiplicity of essentially parallel pipettes which are spaced apart from each other in a pattern corresponding to the pattern formed by the liquid inlets of the synthesis chambers, such that each pipette of the multipipette unit can be positioned opposite a liquid inlet of the synthesis chambers.

8. An apparatus according to claim 7, wherein the number of synthesis chambers in the synthesis chamber unit is preferably an integral multiple of the number of pipettes in the multipipette unit.

9. An apparatus according to claim 7 or 8, wherein the liquid inlets of the synthesis chambers as well as the pipettes of the multipipette unit are preferably arranged in a pattern in which they form the nodal points of a rectangular or square network.

10. An apparatus according to any of the claims 1 to 9, wherein the means for introducing liquid comprise a liquid-introduction unit having liquid-introduction ducts on its lower surface, the number of which liquid-introduction ducts corresponds to the number of synthesis chambers, the liquid-introduction ducts being arranged in a pattern corresponding to the pattern formed by the liquid inlets of the synthesis chambers and being adapted to be positioned opposite the liquid inlets of the synthesis chambers by relative movement of the synthesis chamber unit and the liquid-introduction unit, and wherein the liquid-introduction ducts are connected to a common liquid reservoir within the liquid-introduction unit.

11. An apparatus according to claim 10, wherein the liquid reservoir comprises a liquid cavity with a bottom surface and a downward-facing horizontal abutment surface, and wherein the liquid-introduction ducts are in the form of tubes with essentially identically pointed upper ends and with identically inclined upper end surfaces, and wherein the pointed upper ends of the tubes are all in contact with the horizontal abutment surface while the lower part of all the inclined end surfaces is at a distance from and above the highest part of the bottom surface of the liquid cavity.

12. An apparatus according to claim 10, wherein the liquid reservoir comprises a liquid reservoir with a bottom surface and a downward facing horizontal surface having uniform, preferably substantially hemispherical depressions,
and uniformly dimensioned vertical tubes with upper end surfaces at a right angle to the tube axis, the upper ends projecting uniformly up into the centres of the depressions, but not making contact with the inner surface thereof, and being at a distance from and above the highest part of the bottom surface of the liquid cavity.

13. An apparatus according to any of the claims 10 to 12, comprising guiding means for positioning the liquid-introduction ducts opposite the liquid inlets of the synthesis chamber unit upon bringing together the liquid-introduction unit and the synthesis chamber unit.

14. An apparatus according to any of the claims 10 to 13, comprising moving means for bringing about relative movement of the synthesis chamber unit and the liquid-introduction unit between
an inactive position in which the two units are at a distance from each other, and
an active position in which the two units are in engagement with each other and in which the liquid-introduction ducts of the liquid-introduction unit are positioned opposite the liquid inlets of the synthesis chamber unit.

15. An apparatus according to any of the claims 1 to 14, comprising an electronic control unit for controlling the function of the means for introducing liquid and the means for removal of liquid.

16. A method for multiple peptide synthesis employing an apparatus according to any of the claims 1 to 15, whereby there is provided in each of the synthesis chambers a solid-phase support material having a first, at least *N*-protected amino acid coupled thereto, and whereby a liquid deprotection reagent is introduced into the synthesis chambers by means of the liquid-introduction means, the reagent being removed from the synthesis chambers after completion of the reaction by means of the liquid-removal means, after which a second, at least

N-protected amino acid is introduced into each of the synthesis chambers by means of the liquid-introduction means in order to couple the first and second amino acids.

17. A method according to claim 16, wherein further, at l ast *N*-protected amino acids are introduced successively into the synthesis chambers, said introduction being preceded by introduction of deprotection reagent.

18. A method according to claim 16 or 17, wherein deprotection reagent containing a dye is used.

19. A method according to any of the claims 16 to 18, wherein washing through of the synthesis chambers is carried out after removal of deprotection reagent and before subsequent introduction of amino acid into the synthesis chambers, a liquid washing agent being introduced into the synthesis chambers by means of the liquid-introduction means and being removed from the synthesis chambers by means of the liquid removal means.

20. A method according to any of the claims 16 to 19, wherein there is chosen at least one different first, second or further amino acid for the different synthesis chambers.

21. A method according to any of the claims 16 to 20, wherein deprotection reagent and/or washing agent is introduced into the synthesis chambers from a common reservoir by means of the liquid-introduction means.

22. A method according to any of the claims 16 to 21, wherein amino acids are introduced into the synthesis chambers by means of a multipipette unit.

23. A method according to claim 22, wherein amino acids are transferred to the synthesis chambers from a number of reservoirs containing different amino acids, and wherein the synthesis chambers, whose number is a integral multiple of the number of pipettes in the multipipette unit, are divided into groups of equal size, the number of

which corresponds to the number of pipettes, and wherein amino acids are transferred by means of the multipipette unit from different reservoirs to the synthesis chambers in the various groups.

24. A method according to any of the claims 16 to 23, wherein liquid is prevented from running out of the synthesis chambers via their liquid outlets by introducing a gas under a suitable pressure to the liquid outlets.

25. A method according to any of the claims 16 to 24, wherein liquid is removed from the synthesis chambers by establishing a vacuum in their liquid outlets.

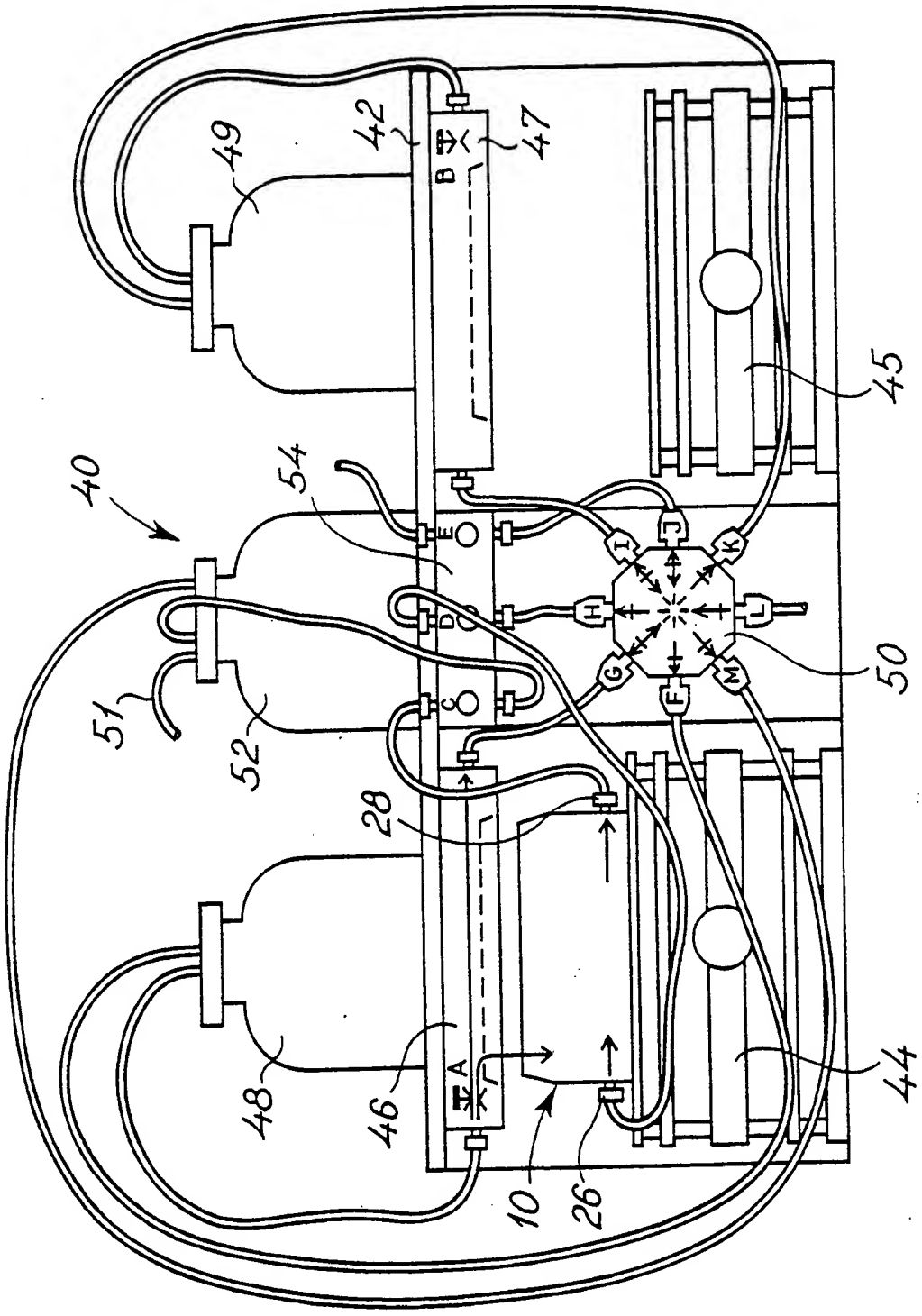


Fig. 1

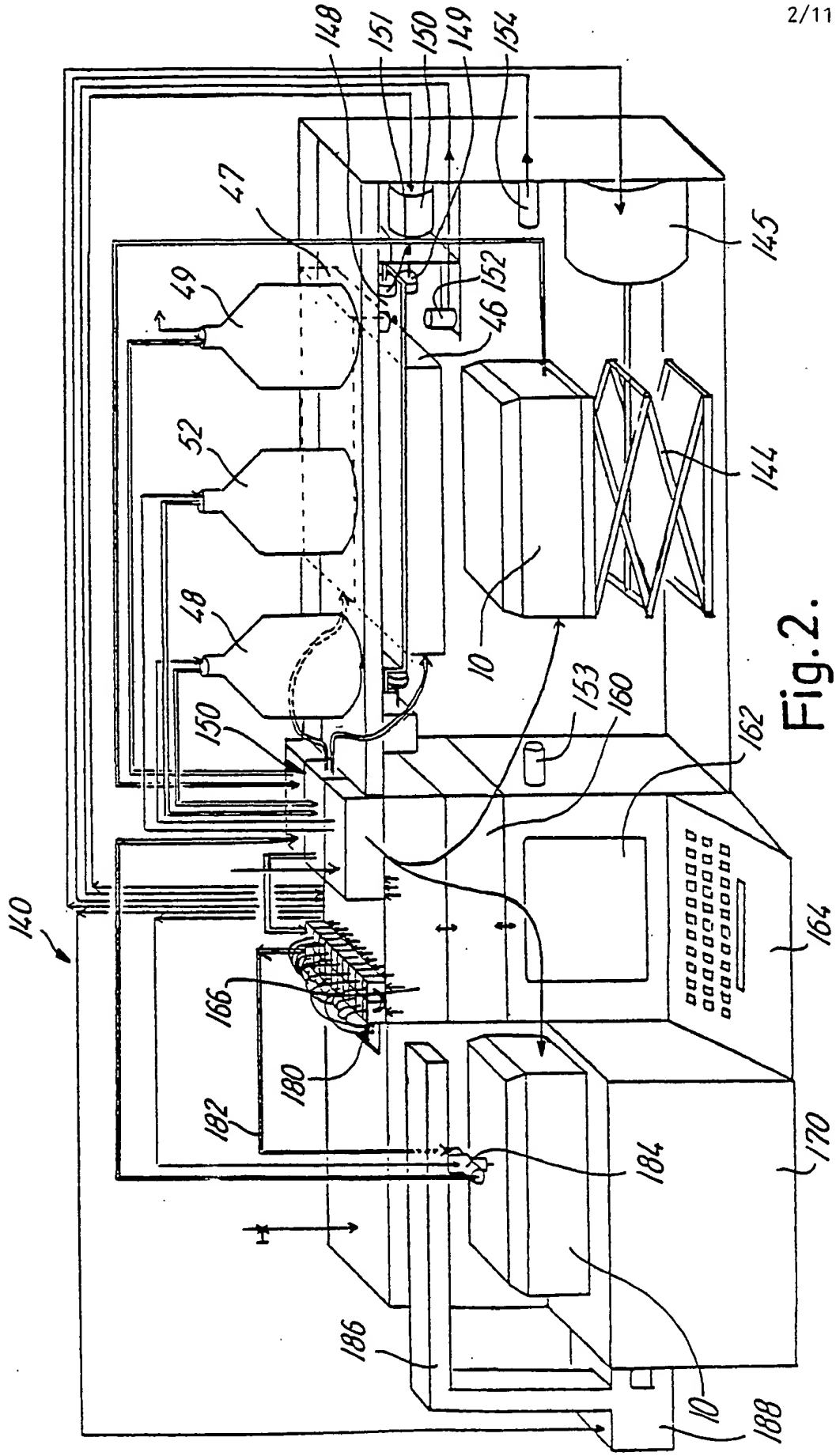

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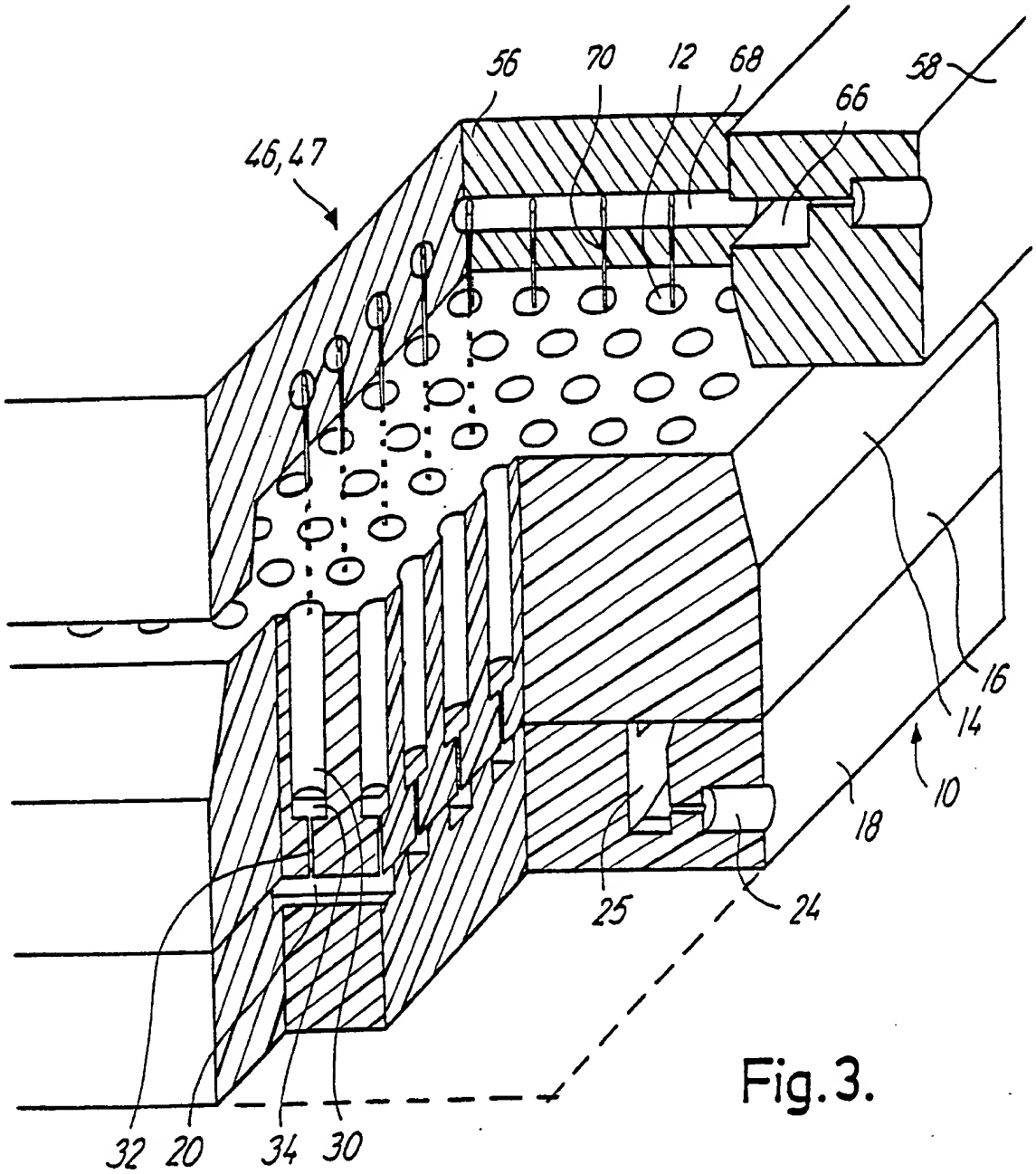
Fig.2.



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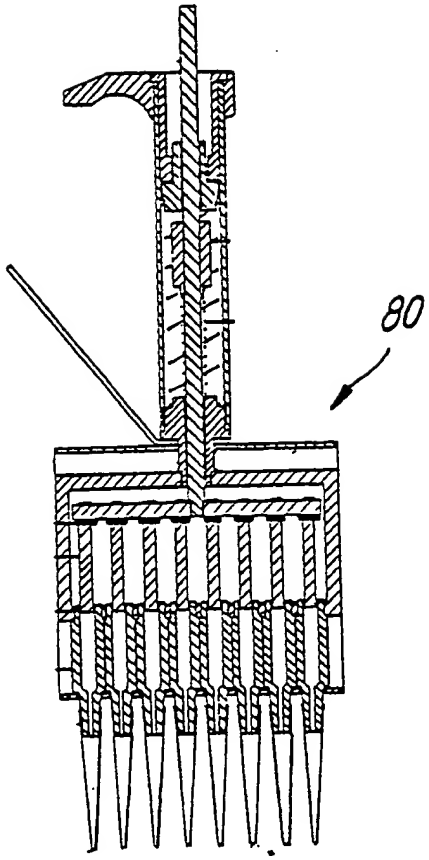

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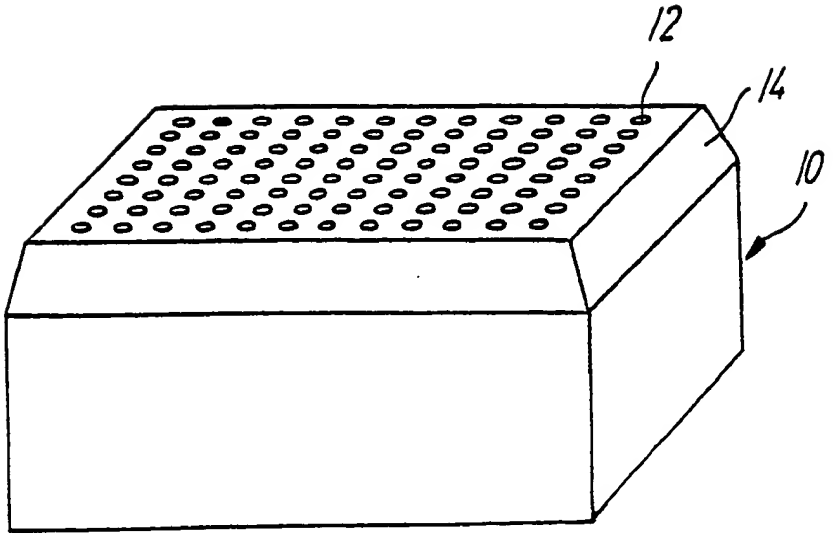

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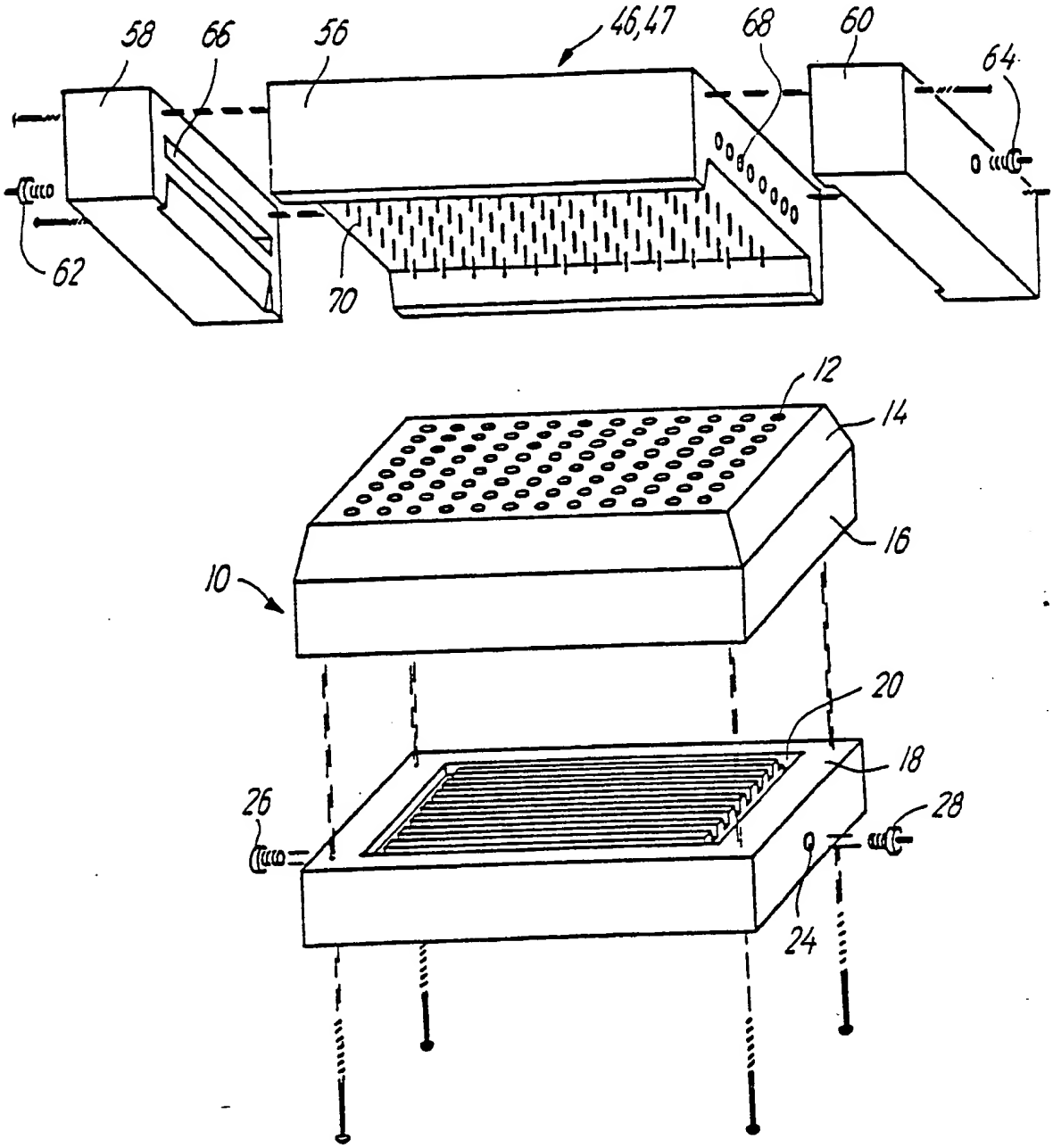

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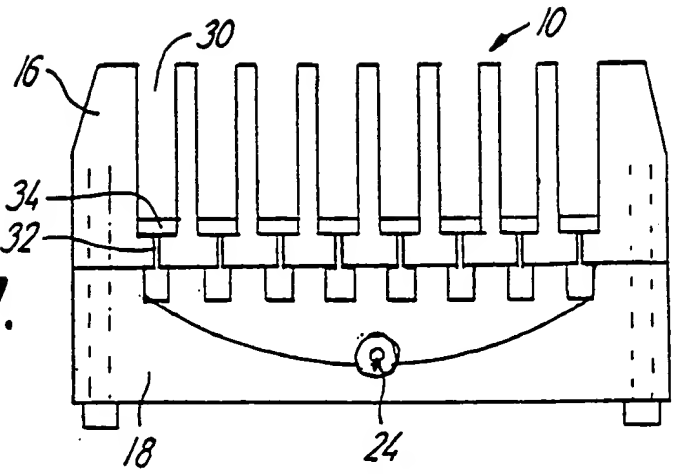

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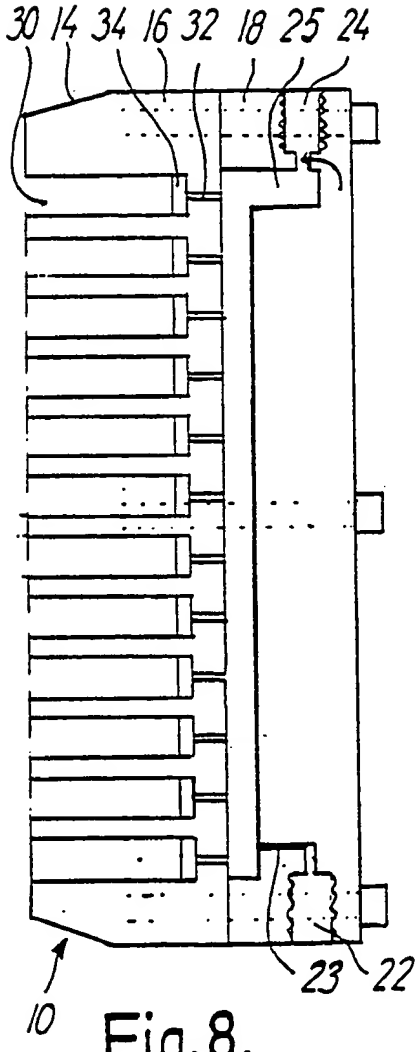

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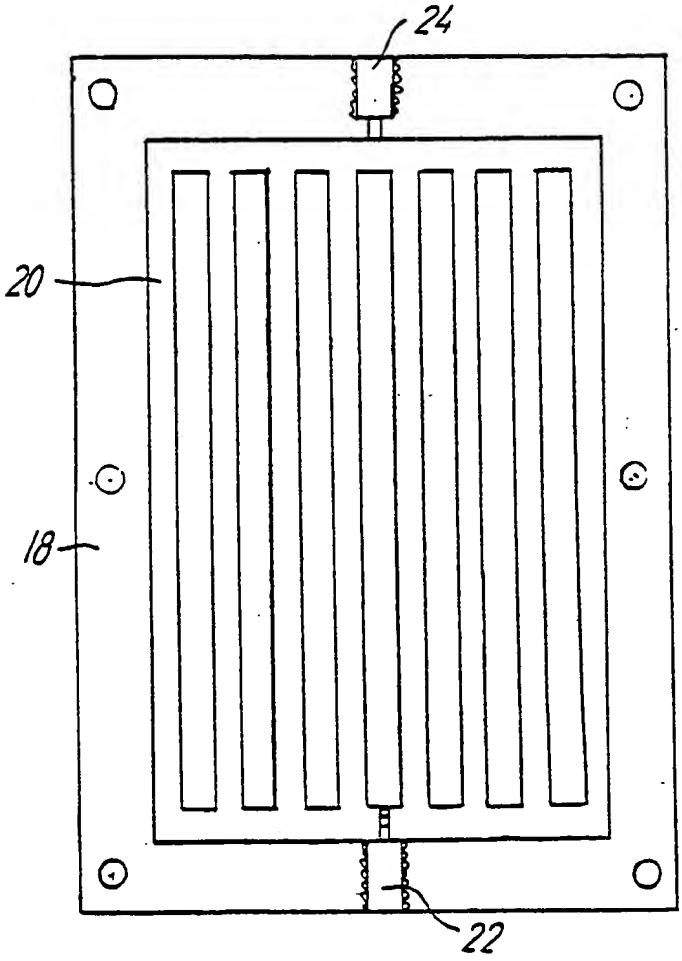

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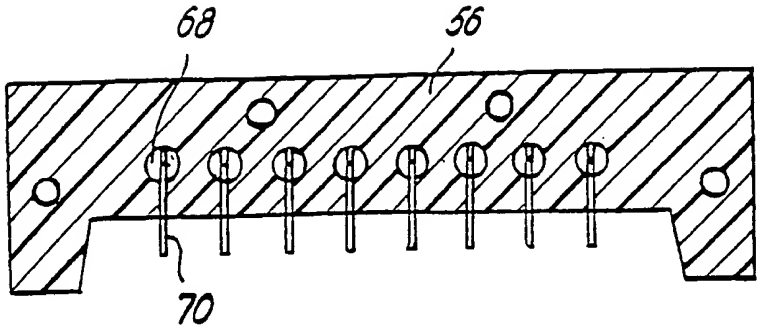
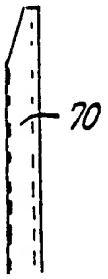


Fig.11.



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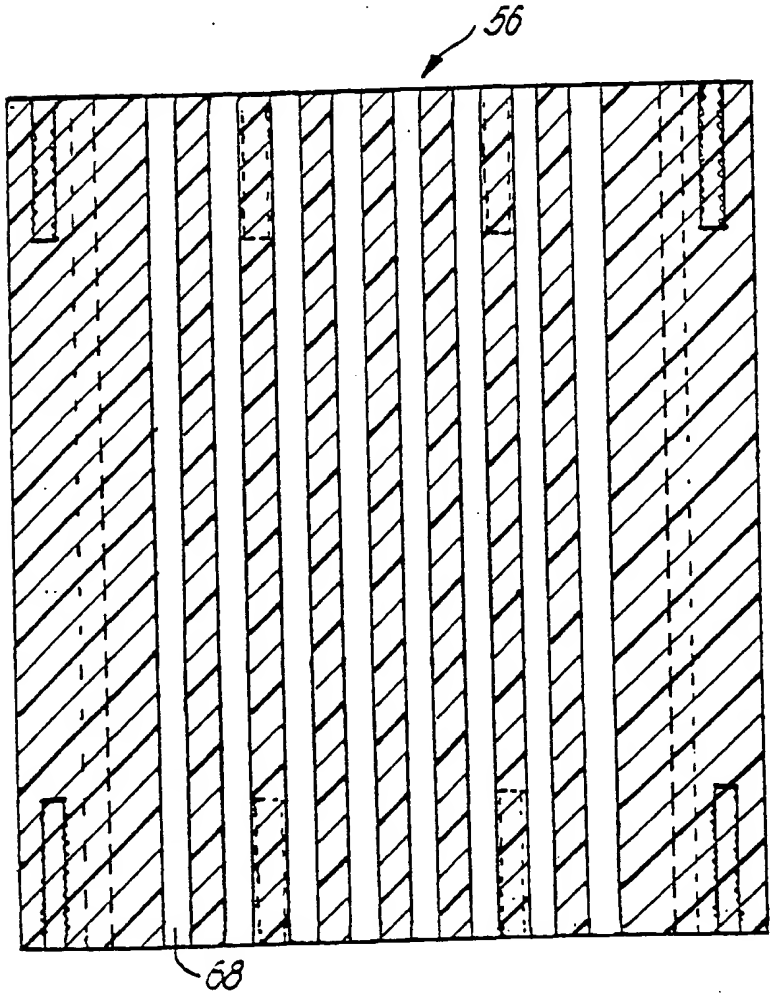

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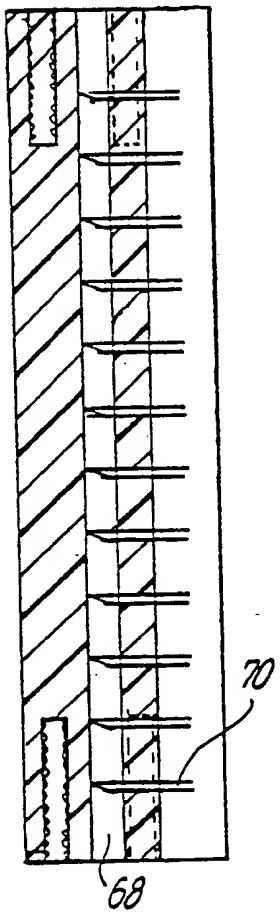


Fig.13.

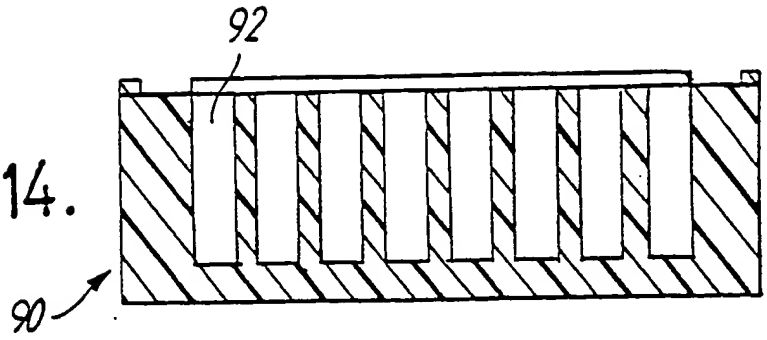


Fig. 14.

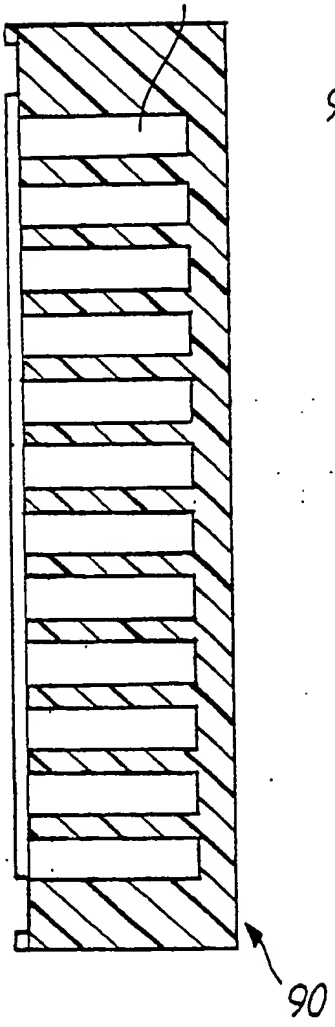
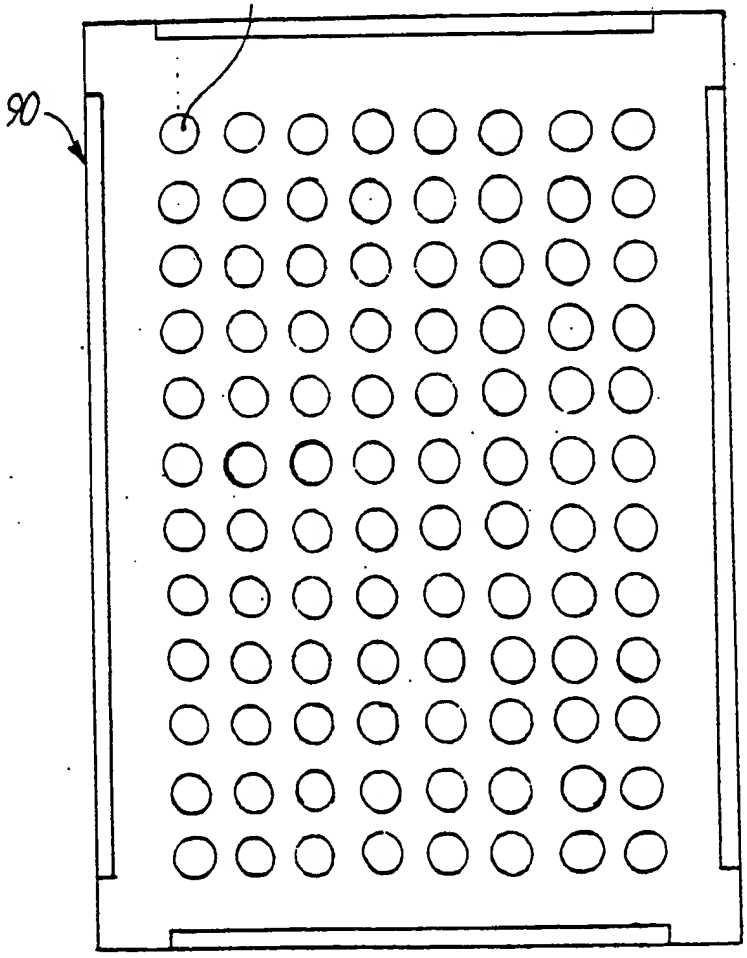


Fig. 15.



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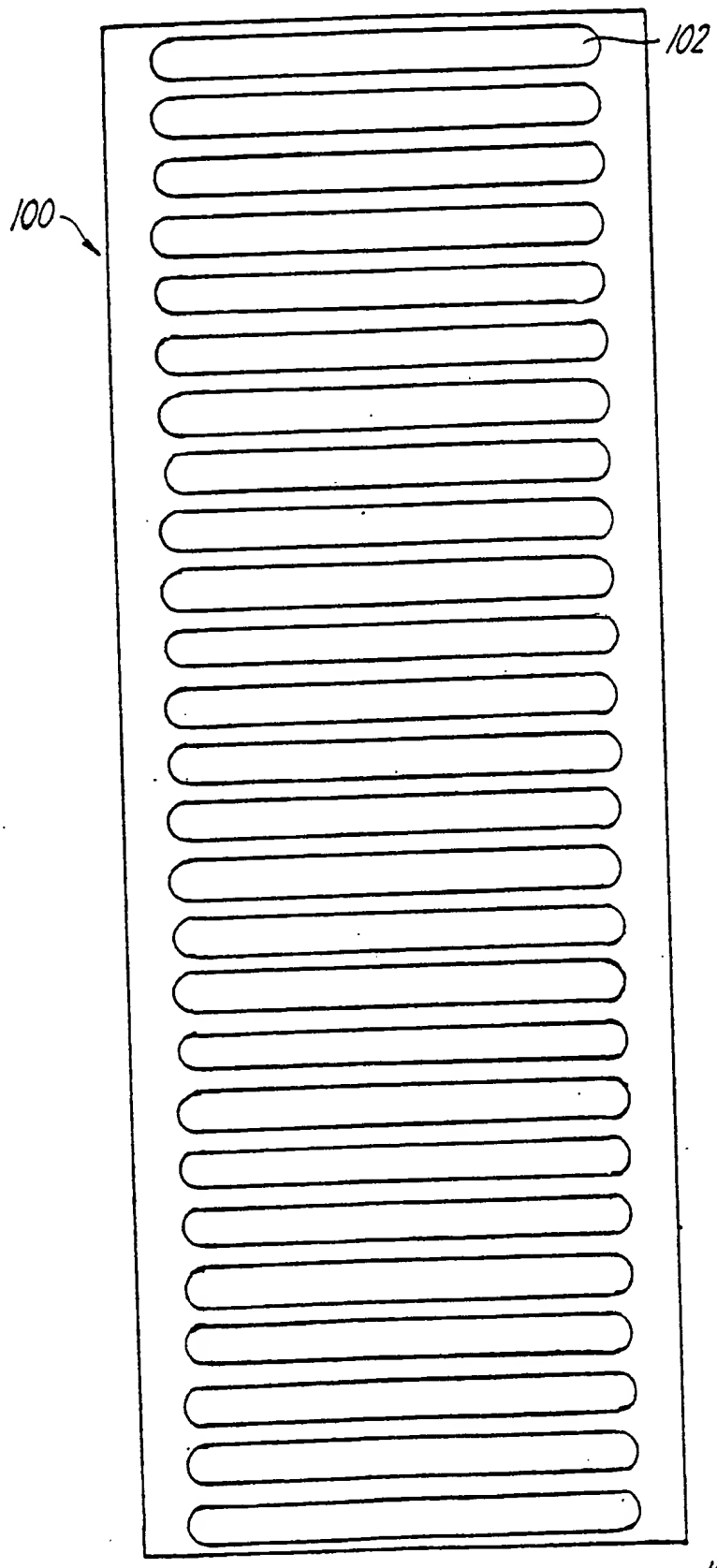

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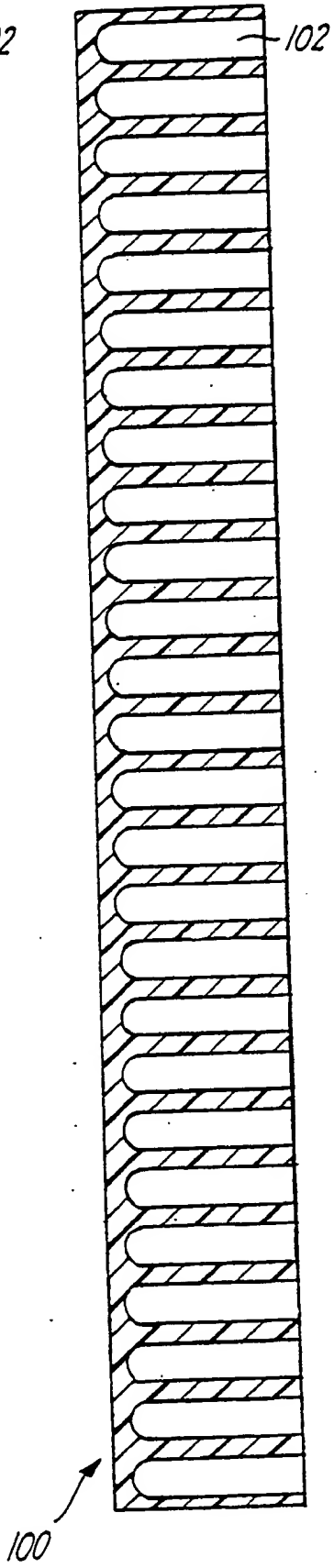
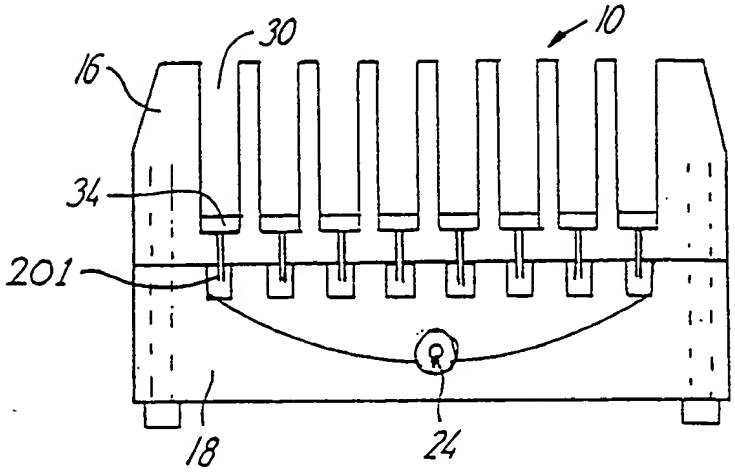
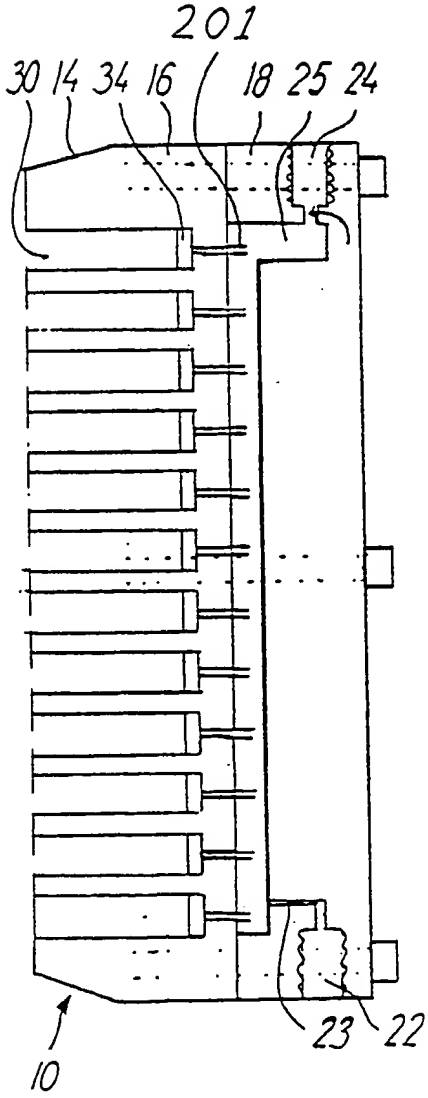


Fig. 18.

Fig.19.





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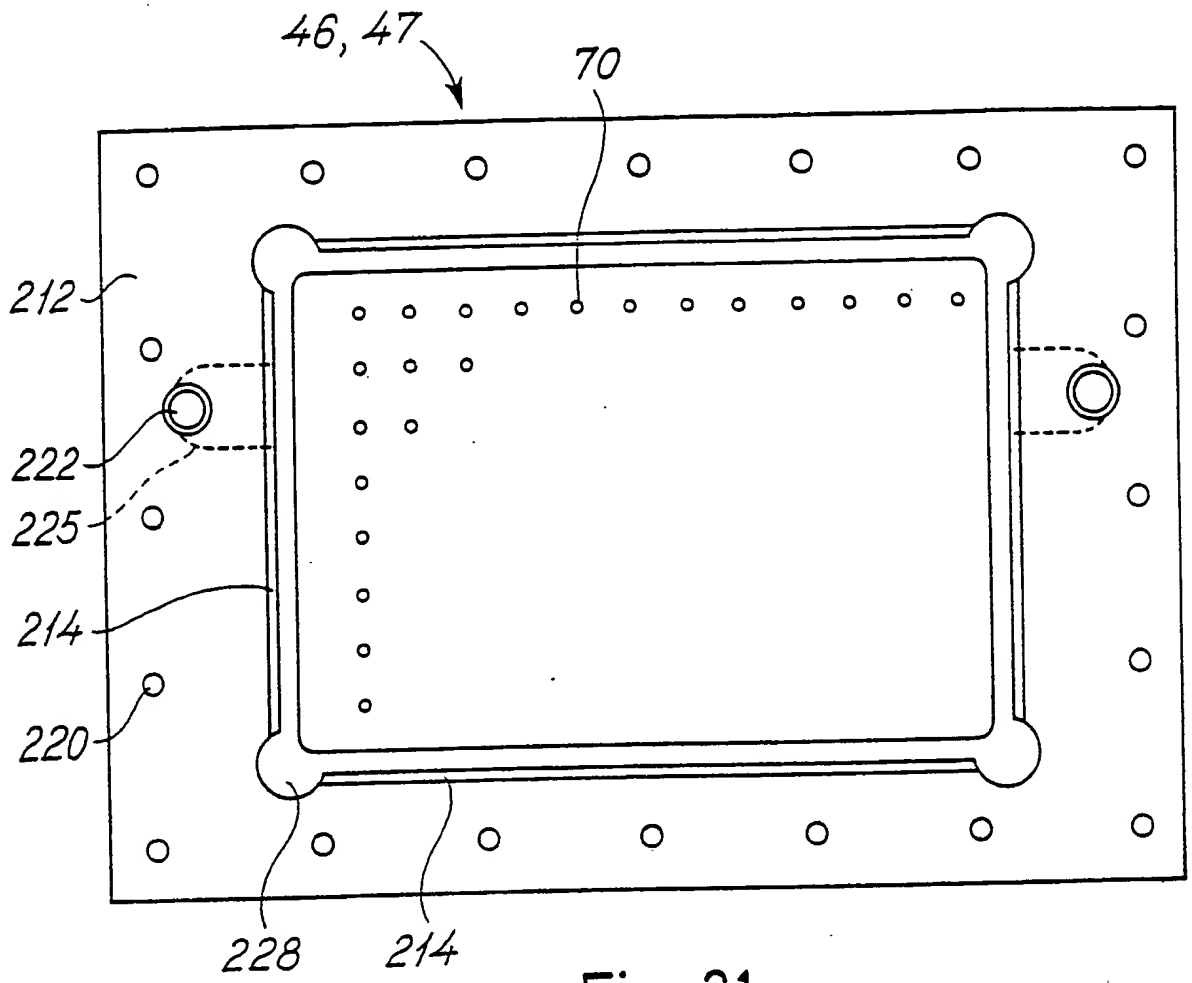
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Fig. 21

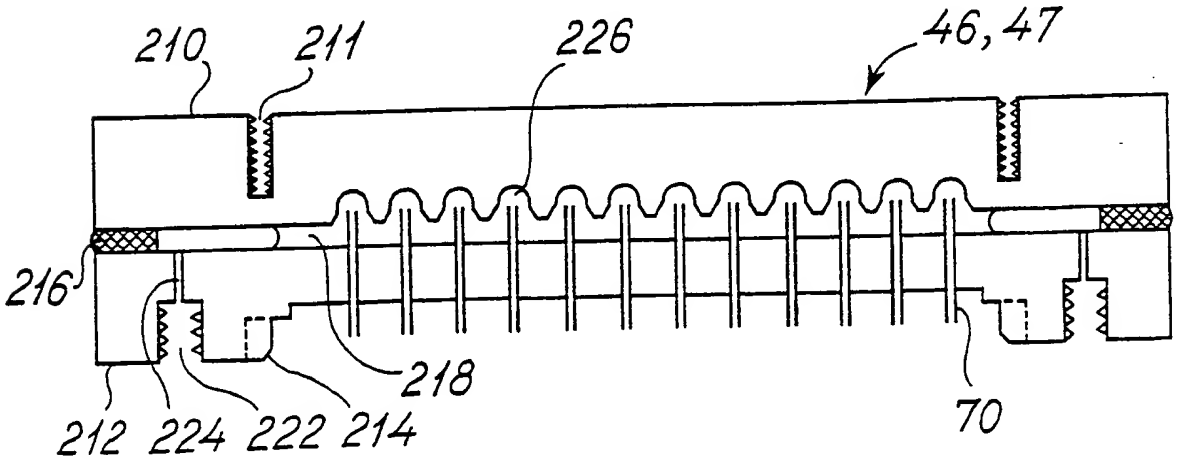
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Fig. 22

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 89/00206

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: B 01 J 19/00, G 01 N 33/543, C 07 K 17/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC4	B 01 J; G 01 N; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	J. Am. Chem. Soc., Vol. 85, July 1963 (New York) R. B Merrifield: "Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide ", see page 2149 - page 2154 --	16-25
A	WO, A1, 87/02138 (LABSYSTEMS OY) 9 April 1987, see the whole document --	1
X	WO, A1, 82/03690 (PHARMACIA DIAGNOSTICS AB) 28 October 1982, see the whole document --	1-10, 14
X	WO, A1, 86/02168 (CETUS CORPORATION) 10 April 1986, see the whole document --	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th December 1989	1989 -12- 14
International Searching Authority	Signature of Authorized Officer [14]
SWEDISH PATENT OFFICE	Eva Iversen Hasselrot

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	WO, A2, 87/06008 (BECKMAN INSTRUMENTS INC) 8 October 1987, see the whole document --	1
X	EP, A2, 0156588 (APPLIED BIOSYSTEMS INC) 2 October 1985, see the whole document --	15
X	GB, B, 1305815 (COOKE ENGINEERING COMPANY) 7 February 1973, see the whole document --	1,8,9, 10
X	US, A, 4701304 (MARCUS J. HORN ET AL) 20 October 1987, see the whole document --	15
X	US, A, 4746490 (HOSSAIN H. SANEII) 24 May 1988, see column 14, line 65 - column 15, line 68 --	1-3,6, 10,15-21,24

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 89/00206

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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